11-2-2010 12:00 AM

The Lotus japonicus cytokinin receptor gene family and its role in nitrogen-fixing symbiosis

Mark A. Held
The University of Western Ontario

Supervisor
Dr. Krzysztof Szczyglowski
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

The *Lotus japonicus* cytokinin receptor gene family and its role in nitrogen-fixing symbiosis

(Spine Title: The *Lotus japonicus* cytokinin receptor gene family)

(Thesis format: Monograph)

By

Mark Anton Held

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Krzysztof Szczyglowski

Co-Supervisor

Dr. Susanne Kohalmi

Advisory Committee

Dr. Denis Maxwell

Dr. Kathleen Hill

Examiners

Dr. Denis Maxwell

Dr. Greg Thorn

Dr. Mark Daley

Dr. Dario Bonetta

The thesis by

Mark Anton Held

entitled:

The *Lotus japonicus* cytokinin receptor gene family and its role in nitrogen-fixing symbiosis

is accepted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Date _____

_____
Chair of the Thesis Examination Board

ABSTRACT

Nitrogen is the most abundant element in our atmosphere, yet has become increasingly limited in agricultural lands. Legume plants offer a possible solution to this problem due to their innate ability to symbiotically interact with nitrogen-fixing bacteria called rhizobia. In particular, a histidine kinase cytokinin receptor from the model legume *Lotus japonicus* (LHK1) has been clearly placed at the core of these interactions. Loss-of-function mutants in LHK1 fail to initiate timely cortical cell divisions in response to abundant bacterial infection, and gain-of-function mutations in the same locus cause *L. japonicus* plants to form spontaneous nodules in the absence of rhizobia, thus indicating that this receptor is required and sufficient for nodule organogenesis. However, nodulation events do still occur in *lhk1-1* mutants. Therefore, this study has sought to address how nodule organogenesis persists in the *lhk1-1* mutant background; is this achieved through cytokinin-independent signaling or perhaps redundancy in function with other members of the LHK family? To that end, the present study has identified three new *Lhk* loci from *L. japonicus* (described herein as *Lhk1A*, *Lhk2*, and *Lhk3*) and provides a detailed characterization of their roles during the NFS. Furthermore, we highlight the pivotal role of LHK1 signalling during the NFS, but also clearly indicate that the role of this receptor is not entirely unique. Indeed, other LHK family members share promoter localization profiles with LHK1 and can complement for loss-of-function mutations in LHK1. The results have allowed for the refinement of current models involving the cytokinin signalling network, which highlight a possible role for other receptors during LHK1-independent signalling events in the root cortex.

KEYWORDS: legumes, *Lotus japonicus*, nitrogen-fixing symbiosis, rhizobia, mycorrhizal fungus, cytokinin, histidine kinase, cytokinin receptor

DEDICATION

To my better half, Megan; who saw me through this journey.

ACKNOWLEDGEMENTS

I am foremost grateful to my supervisor and mentor Dr. Krzysztof Szczyglowski, for his guidance, support and constant pursuit of excellence in science.

I would like to thank the members of my advisory committee, Dr. Denis Maxwell and Dr. Kathleen Hill, and my co-supervisor Dr. Susanne Kohalmi for providing helpful advice and feedback during my graduate studies.

Thank you to all the past and present Szczyglowski lab members who I have shared work, but also jokes with in the lab.

In particular, I would like to thank Dr. Bogumil Karaś for all of his support and technical advice; for working late, many nights discussing new ideas or pushing me to new heights.

I would like to thank the Natural Sciences and Engineering Research Council (NSERC) Postgraduate Scholarship, the Ontario Genomics Institute, and the UWO Biology department for providing me with financial support throughout my graduate studies.

Thank you to all the close friends and family that have given me encouragement along the way; I could not have completed this task without your support.

Lastly, but definitely not least, I thank my parents for making great sacrifices in their own lives so that I may have the opportunity to go this far.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AM	arbuscular mycorrhiza
BAP	benzylaminopurine
B & D	Broughton and Dilworth nutrient media
C	carbon
Ca^{2+}	calcium
CaMV	Cauliflower mosaic virus
CHASE	cyclases/histidine kinases associated sensory extracellular
CI	Confidence interval
CSG	common symbiosis genes
cZ, Cis-Z	*cis*-Zeatin
DNA	deoxyribonucleic acid
EMS	ethylmethanesulfonate
GMO	genetically modified organism
GRAS	GAI, RGA, SCR
H	hydrogen
HA	hectare
HK	histidine kinase
HPT	histidine phosphotransfer protein
iP	isopentenyl adenine
IT	infection thread(s)
kDa	kilodalton
Mb	megabase

MC	microcolony
MMt	million metric tonnes
Mya	million years ago
MF	mycorrhizal factor
N	nitrogen
NAA	1-naphthaleneacetic acid
NF	nodulation or nod factor
NFS	nitrogen-fixing symbiosis
ORF	open reading frame
P	phosphorus
pTZS	tZ secretion plasmid
R	variable group
RACE	rapid amplification of cDNA ends
RcsC	regulator capsule synthesis C
Rht	reduced height gene
RNA	ribonucleic acid
RR	response regulator
RT-PCR	reverse transcription polymerase chain reaction
SCAR	suppressor of cAMP receptor defects
Tg	teragram (equivalent to MMt)
TILLING	targeted induced localized lesions in genomes
TM	transmembrane domain
tZ	*trans*-Zeatin

UBI	ubiquitin
UTR	untranslated region
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein

All numerical units included in this thesis are standard SI units

CHAPTER 1

INTRODUCTION

1.1 The green revolution

The transition from hunter-gatherer lifestyles to agriculture coincided with the domestication of both plants and animals. Historically, this was most likely to occur in geographic locations such as the Fertile Crescent (Western Asia), where, some 10 000 years ago, indigenous peoples were surrounded by a local abundance of usable plants such as wheat, barley and peas as well as what would become livestock animals such as sheep, goats, cows and pigs (Diamond, 2002; Lev-Yadun *et al.*, 2000). Since that moment of inception, human agriculture practices have and continue to shape the global landscape of Earth. Among the advances made since that time, the so-called 'Green Revolution' of the mid twentieth-century was likely one of the most significant. Originally pioneered by the work of Norman Borlaug, the green revolution combined improved crop varieties, such as dwarf varieties of wheat containing altered forms of the giberellin-insensitivity genes (*Rht*; Smale, 1997), with refinement of pre-existing technologies such as irrigation and the development of novel technologies like agrochemicals in the form of synthetic fertilizers (Khush, 2001). The combined effect of these variables significantly increased the productivity of agricultural lands the world over (Fig. 1.1). For example, the semi-dwarf IR8 rice strain produced by the International Rice Research Institute (IRRI) provided exceptional yields under sufficient fertilizer and irrigation regimes. IR8 represents a quintessential green revolution crop which significantly contributed to vast increases in agricultural output from developing and developed countries alike (Flinn *et al.*, 1982; Khush, 2001). For example, agricultural output doubled from developing nations between 1965-1981, largely as a

result of increased yields obtained from cereal crops such as rice, wheat, and corn (Fig. 1.1; Foley *et al.,* 2005).

The vast majority of crops grown globally are now genetically-modified organisms (GMO) that have been engineered to exhibit, among other desired traits, increased resistance to herbicides, insecticides, and pests of various types (Conner *et al.*, 2003). For example, in China improved varieties of wheat, rice and corn represent nearly 100% of all crops planted (Huang *et al.*, 1999). In addition to these genetic variables, a great deal of investment has been made in maximizing yields of many crop species closer to those obtained in artificial lab settings, where conditions are ideal. At the time of the green revolution researchers became very aware of this fact and thus, novel GMO crops used in modern agriculture depend heavily on nutrient status, particularly that of nitrogen (N) and phosphorus (P).

Phosphorus and phosphate-containing fertilizers are obtained via intensive mining of limited deposits found in a few, specific locations like Canada. A large portion of extracted P is applied to agricultural lands to supplement soil limitations, which affect a significant portion of the arable land globally (Vance *et al.,* 2003).

Elemental N comprises roughly 78% of the Earth's atmosphere. However, N atoms in this state are unusable by nearly all organisms due to the triple bond connecting the two N atoms, which makes the conversion of dinitrogen into other compounds extremely difficult. This is particularly significant when one considers that N is the most limiting factor for terrestrial crop growth and yield. The production of nitrogenous compounds from hydrogen and N gas was pioneered by Fritz Haber and Carl Bosch, who were awarded Nobel prizes in 1918 and 1931, respectively, for their refinement of

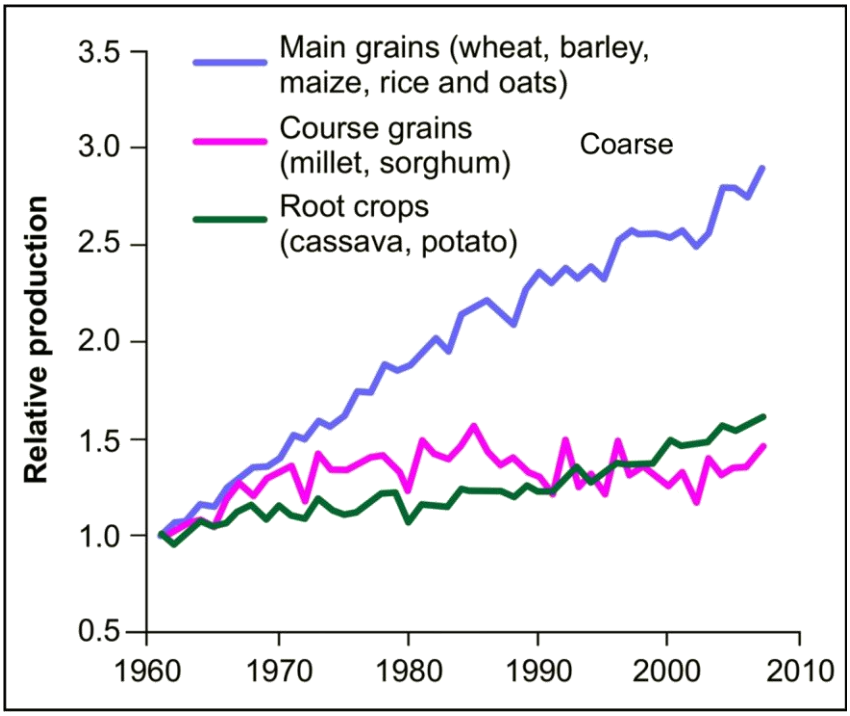


Figure 1.1 Changes in global land use strategies over time. (A) Agricultural lands have gradually transitioned from natural landscapes into areas largely dominated by intensive agriculture. (B) Increases in crop production during and after the industrial and green revolutions of the 20th century. Figure modified from Foley *et al.*, (2005).

industrial N fixation into an economically feasible process, aptly named the Haber-Bosch process. This industrial N fixation consumes 1% of the total energy budget to produce roughly 100 million tons of largely anhydrous ammonia per year, which is mostly used for the production of N fertilizer (Smith, 2002).

On a global scale, N from the Earth's atmosphere prior to the industrial and green revolution has been contributing to overall N cycle through natural processes, such as a combination of biological N fixation by bacteria and cyanobacteria inhabiting terrestrial and aquatic environments, lightning strikes and symbiotic N fixation of leguminous plants. At this time, very little in the way of anthropogenic (human-derived) N fixation contributed to the global N fixation quotient (Fig. 1.2). However, since the green and industrial revolutions of the mid-20th century, the global balance of N inputs has shifted significantly due to humanity's increasing combustion of fossil fuels and our increasing employment of the Haber-Bosch process; up from only 10.3 MMt (million metric tones) to a projected 240 MMt by 2050 (Tilman, *et al.*, 2002; Vance, 2001). Over half of the total global N fixation budget is now derived from anthropogenic sources, with industrial N fixation used in the production of N-based fertilizers comprising the majority of that number (Fig. 1.2).

1.2 Consequences of intensive agriculture

In spite of the great advancements made by the green revolution and the countless number of human lives saved because of increases in the global production of cereal crops, there are a significant number of detrimental side-effects which we are only now beginning to comprehend including, health effects relating to pesticide usage, water

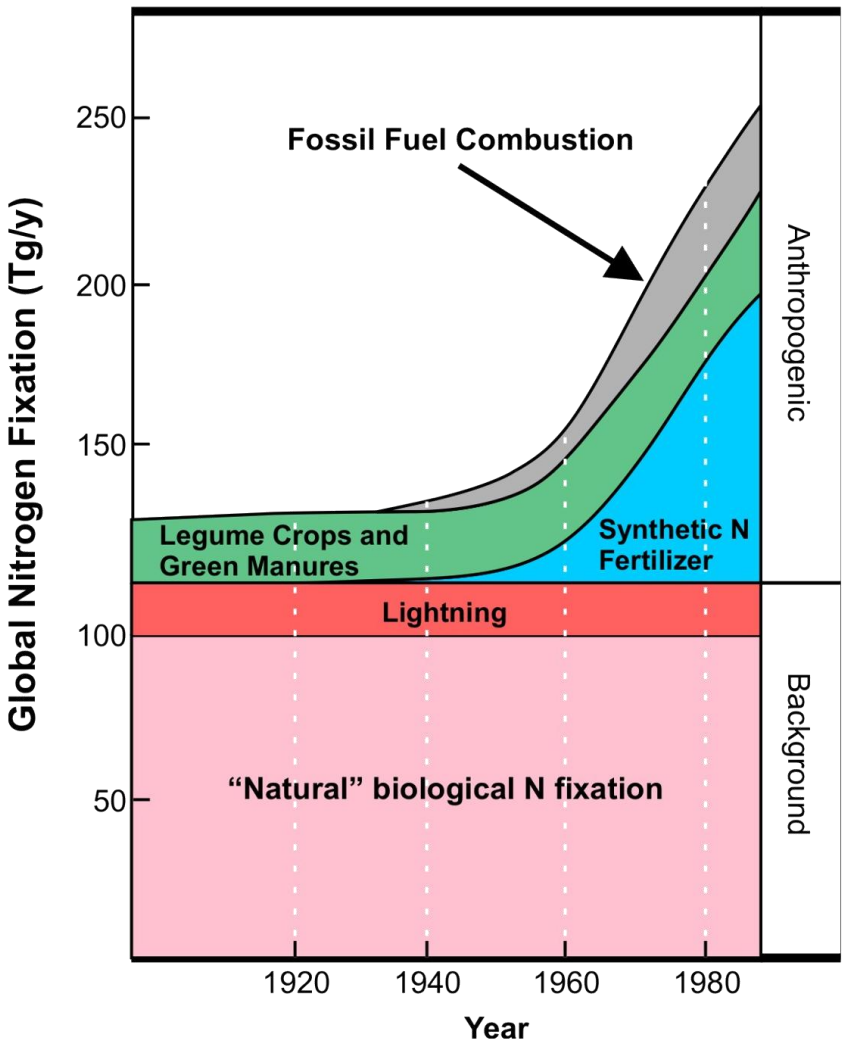


Figure 1.2 Global N fixation during the 20th century. The proportion of natural to anthropogenic N fixation has significantly changed with increasing demand for nitrogenous fertilizers as a result of the green revolution. Figure modified from Vitousek and Matson (1993).

shortages and massive salination of once arable land, extensive loss of soil fertility and biodiversity, habitat loss due to the conversion of natural landscapes into agricultural land and the eutrophication of aquatic environments due to leaching of N and P-based fertilizers from agricultural lands (Foley *et al.,* 2005; Godfray *et al.,* 2010). In addition, non-renewable sources of phosphate are expected to vanish within the next 40 years, based on current extraction rates (Vance *et al.,* 2003).

One of the greatest contributors to the nitrogen problem is the fact that a large portion of the applied compound is not utilized by the plant. For N, it has been estimated from early reports that (depending on the crop) only up to 50% of applied fertilizer is recovered by the plant and for P this number is much lower at 5 to 25% (Papendick *et al.,* 1986). This results in not only the accumulation of N and P within agricultural soils, but also in the subsequent inundation of aquatic, terrestrial and atmospheric environments with excess nitrate and phosphate. For example, recent modeling of the correlation between maize yield and nitrate leaching has indicated that increased nitrate fertilizer application will lead to a drastic increase of leaching into groundwaters of the upper Mississippi river basin, while only providing marginal yield increases (Fig. 1.3).

Agriculture is now the largest source of excess N and P in aquatic environments (Bennett *et al.,* 2001). This excess nitrate and P are known to have a number of detrimental effects on human health and the environment. In the case of P, it has been estimated recently that the P storage value for terrestrial and freshwater ecosystems has increased 75% from pre-industrial revolution values (Bennett *et al.,* 2001). This scenario

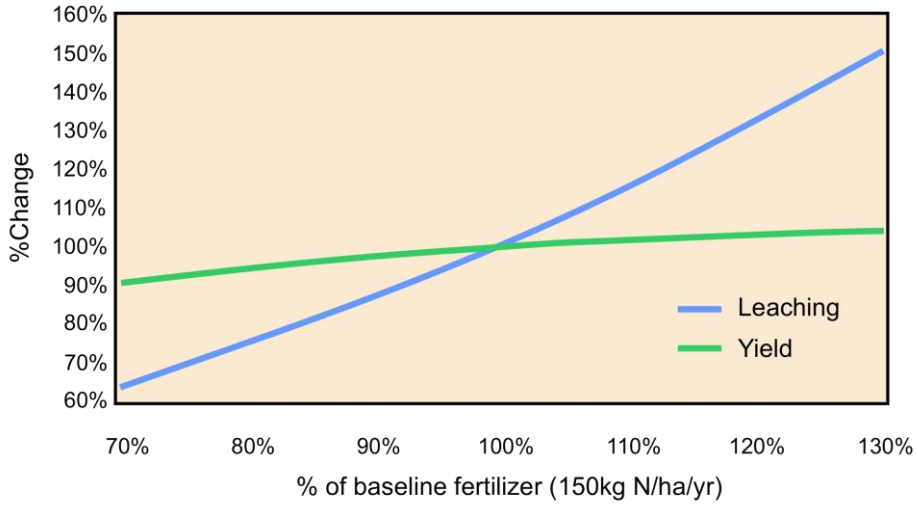


Figure 1.3 Changes in maize yield and nitrate leaching. Forecasted changes are shown as a function of fertilizer application. Figure modified from Foley *et al.*, (2005).

is compounded by the fact that early work by Schindler (1977) clearly indicated that P input is the driving factor for primary production in freshwater lakes, which leads to large-scale algal blooms in freshwater systems, reduced oxygen content and fish kills (Bennett *et al.,* 2001; Carpenter *et al.,* 1998). The eutrophication of freshwater resources in the United States due to nitrate leaching and P run-off is believed to have produced the majority of so-called 'impaired' lakes and river systems (Carpenter *et al.,* 1998). Impairment in this context implies that a given body is unsuitable for recreational activities including fishing and boating, as well as irrigation, and use as drinking water (Carpenter *et al.,* 1998; Foley *et al.,* 2005; Matson *et al.,* 1997). Moreover, hypoxic conditions affect vast stretches of coastal areas as algae and other primary producers proliferate in excess nutrient conditions. These so-called 'dead zones,' where the dissolved oxygen content is below levels required to sustain benthic fauna (Diaz and Rosenberg, 2008), are believed to affect nearly 250, 000 square kilometers of aquatic environments worldwide.

In addition to widespread eutrophication, N leaching can affect human health directly. High levels of nitrate in drinking water has been linked to methemoglobinemia; a condition affecting infants three to six months of age. In this age group, bacteria found in the digestive tract can reduce nitrate into nitrite, leading to the accumulation of harmful methemoglobin and therefore, sequestering of oxygen-carrying hemoglobin in the blood stream (Carpentier *et al.,* 1998). The solution to this problem is complex, and likely requires the integration of several factors to be successful. Early reports have suggested a combined approach employing genetic improvement of crop species for greater nutrient acquisition and use, as well as restrictions on the amount of N- and P-

based fertilizers applied to agricultural land (Papendick *et al.*, 1986) and these variables have been echoed by more recent publications (Godfray *et al.*, 2010; Tilman *et al.*, 2002).

1.3 Legumes as an alternative

Farmers are now faced with a complex problem; N and P limitations affect the vast majority of arable land, so to maintain yields, an ever-increasing amount of fertilizers are applied, which result in further environmental degradation as outlined above. It is clear that we must look for alternative means to increase acquisition and/or use efficiency of both N and P. Fortunately, plants have evolved a number of mechanisms to deal with nutrient limitations as an adaptation to their sedentary growth habit, which can be grouped into two broad categories related to (i) enhanced nutrient uptake and (ii) conservation of nutrient use (Vance, 2001). Many researchers believe that the solution to the problem of N and P cycling might be solved in part by increasing our understanding of leguminous plants.

Legumes benefit the environment because of their vast contribution to the natural input of N to soil environments and their ability to thrive under N- and P-limited soil conditions, a talent which is achieved by symbiotic interactions with both phosphate-acquiring arbuscular mycorrhizal (AM) fungus of the phylum Glomeromycota (Parniske, 2008), and N-fixing gram-negative soil bacteria, which are commonly referred to as rhizobia (Oldroyd and Downie, 2008). In more general terms, of the 380 or more angiosperm families known, nodulation is restricted to a single clade of Eurosid I, (Kistner and Parniske, 2002). Although N-fixing symbiosis (NFS) is not present in all genera that belong to this clade, the four orders, namely Fabales, which include legumes,

Fagales, Cucurbitales and Rosales, contain all of the known plant species which form symbiotic, N-fixing interactions. That is, except for one rare relationship known to exist between *Gunnera* (Gunaraceae), commonly known as giant rhubarb, and N-fixing cyanobacteria such as *Nostoc* (Adams and Duggan, 2008).

Legumes, in addition to their significant, N-based contributions to the rhizosphere, provide also for a significant portion of the human diet, including dietary protein, fiber and essential nutrients (Graham and Vance, 2000). Given their overall economic as well as environmental importance, a need arose for the development of model systems which could be use to understand and subsequently exploit their useful and often unique biological properties. Study of the NFS of legumes with rhizobia constituted a major driving force behind the initial selection of model legumes, such as *Lotus japonicus* and *Medicago truncatula*. The immediate aim here has been to rapidly advance our understanding of genetic elements that regulate the symbiotic interaction and also to define the factors that have contributed to the evolution of this useful relationship in such a limited number of plants.

The model legumes have emerged as an attractive platform for this purpose due to their relatively small (~425Mb) diploid genomes, short generation time, ease of transformation, whole-genome sequence databases (Sato *et al.*, 2008; Young and Udvardi, 2009) and forward and reverse genetic resources (Szczyglowski and Stougaard, 2008).

1.4 Evolution of the symbiotic program

Symbiotic interactions have improved the viability of terrestrial plants since their transition from aquatic environments during the Devonian period, more than 450 million

years ago (mya). The most elegant of all symbiotic interactions are those which involve the intracellular accommodation of microsymbionts by the host plant. One of the earliest intracellular symbiotic interactions of plants and fungi has been observed in the fossil record and its origin was postulated to coincide with the transition of plants into the terrestrial landscape and the evolution of root-like structures on primitive plants like mosses, liverworts and other bryophytes (Bonfante and Genre, 2010). This so called AM symbiosis is ancient and also the most promiscuous of all known intracellular symbiotic interactions in terrestrial plants. It has been presumed to persist in more than 90% of all extant land plants (Bonfante and Genre, 2010), but more reasonably only accounts for 65-70% of land plants (Brundrett, 2004 and references therein). The symbiotic partners in this case are AM fungi of the genera *Glomeromycota*, which, among other beneficial properties, serve to increase phosphate uptake by the host plant from the soil (Parniske, 2008).

In a more recent event (ca. 60 mya), a very confined group of flowering plants evolved the ability for intracellular symbiosis with N-fixing soil rhizobia, a beneficial interaction which allows plants to grow independent of N status in the soil. The NFS is therefore characterized by the intracellular accommodation of beneficial rhizobia within specialized root-derived organs, called nodules, where the bacteria actively convert atmospheric N to ammonia and in return, are provided with (among other items) carbon sources by the host plant (Fig. 1.4). There are a number of strikingly similar features of these two symbiotic interactions, which has lead many researchers to the proposition that the more derived NFS, evolved in part via the recruitment of functions once specific to

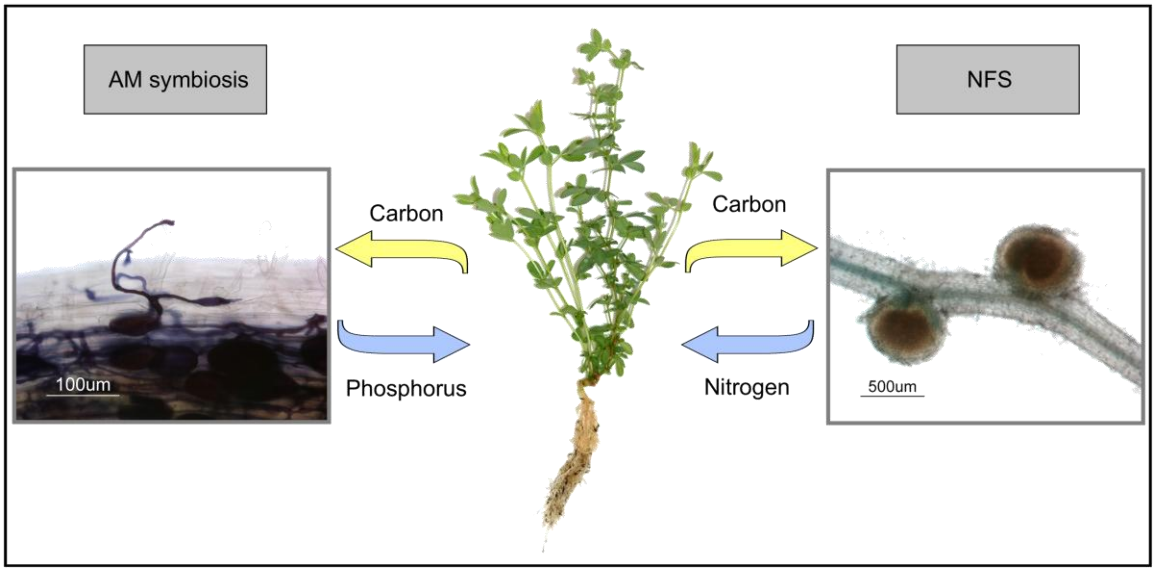


Figure 1.4 Nutrient transfer during legume symbioses. Carbon sources are cycled from the plant's photosynthetic reactions to both fungal (AM symbiosis) and bacterial (NFS) partners in return for P or N, respectively.

the AM symbiosis (Amyot and Szczyglowski, 2003). Firstly, both symbioses rely on chemical signals for their initiation, which are structurally similar. Nodulation or nod factors (NF) have been known for quite some time as variants of a chitin molecule; these are produced by canonical gene clusters in many rhizobial genera in response to isoflavonoid secretion by the host root into the rhizosphere (Oldroyd and Downie, 2008). The mycorrhizal-specific or 'Myc factor' (MF) on the other hand, has eluded discovery for quite some time. However, a patent recently described the structure of the MF (Dénarié *et al.,* 2010*;* patent No. WO2010/049817 A2). It is very reminiscent of NF; however, it is simpler in structure and likely less variable than the more derived molecule utilized during the NFS (Dénarié *et al.,* 2010. Patent # WO2010/049817 A2). Gross comparison of these two molecules indicates similarity in their basic structure and in some effects that they exert on the host plant. The complexity of NF, as defined by a variety of strain-specific modifications present at the reducing and non-reducing end of the chitin backbone of this molecule, was likely an adaptation from the rudimentary chitin backbone of the MF (Fig. 1.5). Secondly, both NF and MF are responsible for the initiation of symbiotic signaling through a subset of well-defined downstream plant genetic elements, which are shared by both symbioses (See section 1.6).

Lastly, both fungi and bacteria are actively accommodated by the host root, which build similar structural features generally referred to as 'cytoplasmic bridges', which guide the symbiont towards the root cortex (Genre *et al.,* 2005). Since early in their evolution, the complexity of interactions between legumes and rhizobial bacteria must have evolved quite significantly, which required at least two key adaptations; (i) intracellular accommodation of the bacteria and (ii) the production of a structure to house

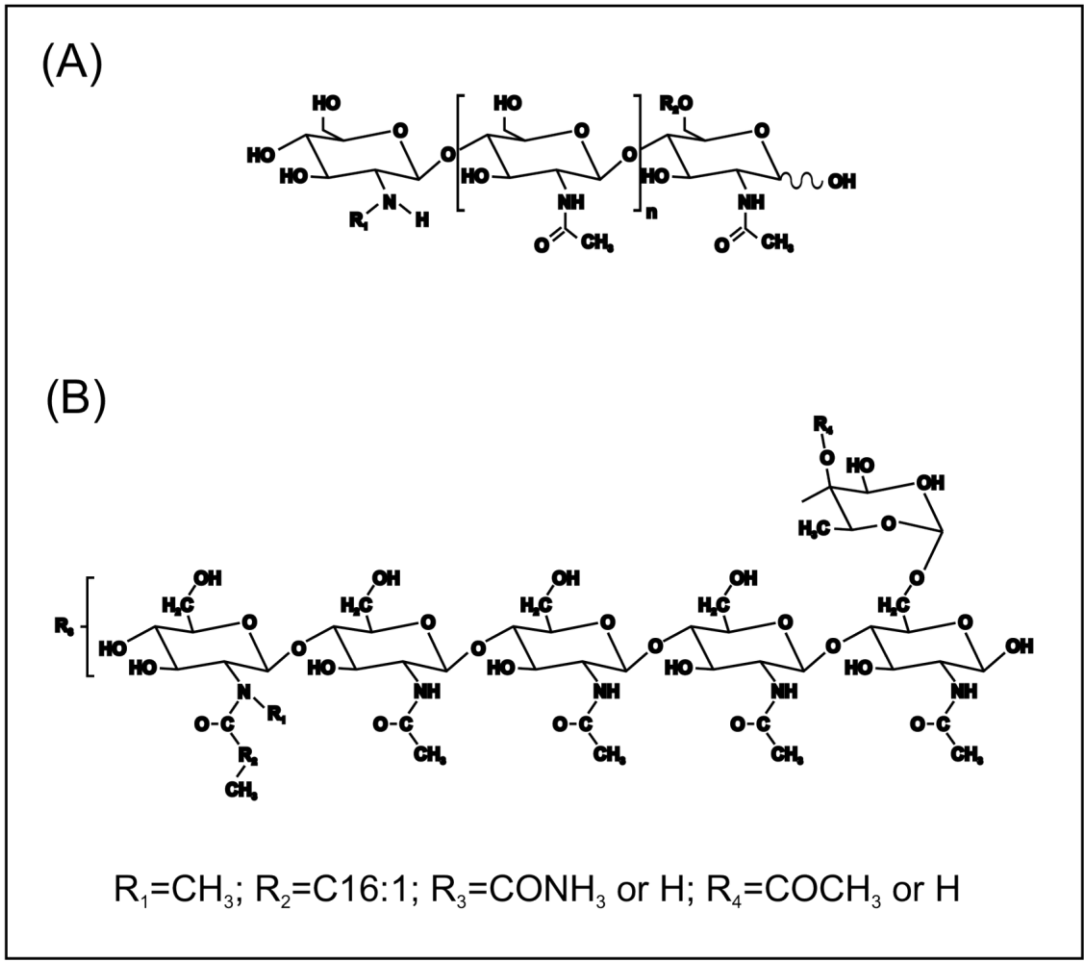


Figure 1.5 Chemical stimuli for beneficial symbioses. (A) Generalized structure of the proposed MF (Danarie *et al.*, 2010. Patent # WO2010/049817 A2) and (B) the more derived, lipochito-oligosaccharide NF of the NFS (figure modified from Niwa *et al.*, 2001).

those bacteria; namely the nodule (Sprent and James, 2007).

In an evolutionary context, more rudimentary plant–bacteria interactions are believed to represent a 'default' state, where bacteria remain restricted to the surface or enter inside the root through cracks while remaining extracellular (Sprent and James, 2007) Direct intracellular entry of bacteria within the root cortex has been recognized in limited cases (Giraud, *et al*. , 2007). However, a more complex scenario exists, which is observable in majority of herbaceous legumes, including *L. japonicus* (Sprent and James, 2007). In this scenario, rhizobia enter root hairs at the epidermis through plant plasma membrane-derived tubular structures called infection threads (IT). They are then shuttled through this conduit to the root cortex in a highly controlled manner, which is set by the host plant (Karas *et al.*, 2005).

1.5 Two programs are required during the NFS

The sophistication of symbiotic N fixation to incorporate bacterial entry and nodule organogenesis has been well-described by genetic and physiological analyses, which span a number of decades. Firstly, bacterial entry is dictated by the epidermal program, which encompasses all processes related to the initial chemical cross-talk between the symbionts, entry and internalization of the rhizobia. In wild-type plants, NF perception leads a number of cellular responses including the entrapment of bacterial microcolonies within root hairs curled in a typical 'Sheppard's crook' conformation (Fig. 1.6A; Gage, 2004). Subsequently, the tonoplast of root hair cells invaginates in a polar-tip growth fashion, which directs bacteria to the base of the epidermal cell in the plant-derived IT.

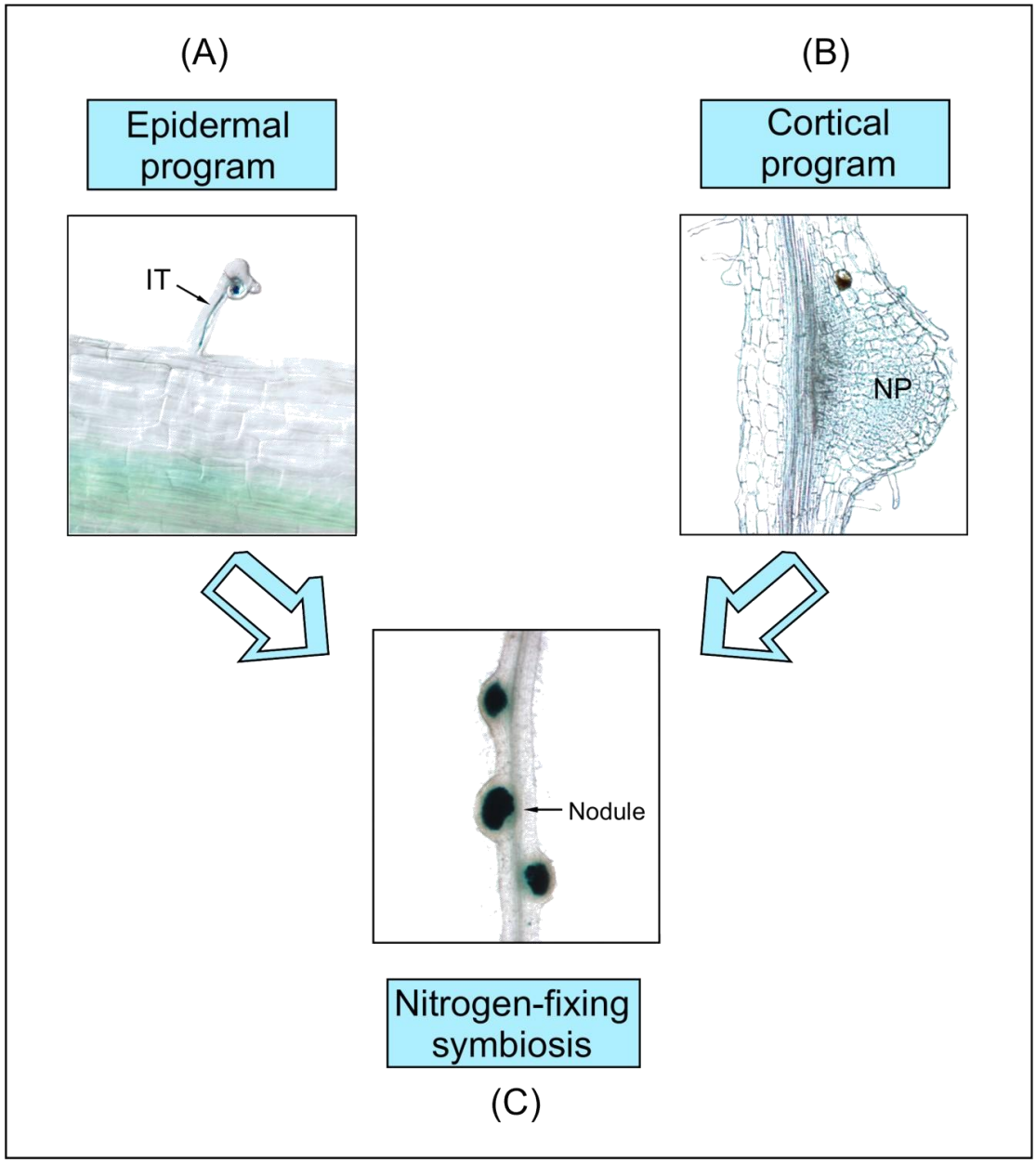


Figure 1.6 Two programs are required for the NFS. (A) The epidermal program regulates bacterial entry and the formation of infection threads, while the cortical program (B) is responsible for cell divisions which produce the nodule primordia. In wild-type plants, bacteria are eventually released into nodule cortex, thus forming a functional, N fixing nodule (C). Rhizobia carrying a *hemA::LacZ* cassette are visualized as a blue color following histochemical staining for *β*-galactosidase activity. IT, infection thread; NP, nodule promordium; Nodule, colonized nodule (arrow).

Once past the epidermis, ITs ramify within the root cortex, the process that is dictated by cytoplasmic bridges laid down by the host plant; this typifies the epidermal program in derived legumes such as *L. japonicus*. A wide range of mutant lines have been identified in *L. japonicus* and other model systems which are affected in their ability to achieve epidermal entry due to genetic lesions in a number of loci (Held *et al.*, 2010). In particular, a group of root hair mutants was recently characterized from *L. japonicus* which elegantly portrayed the importance of the epidermal program, and also discovered alternative means for bacterial entry when the regular development of the root epidermis is disrupted (Karas *et al.*, 2005).

Bacterial perception at the surface of root epidermis also leads to drastic changes within the root cortex where anti- and peri-clinal cortical cell divisions give rise to the nodule primordium (Fig. 1.6B). Simple, yet powerful; the nodule provides the bacteria with not only space, but also, maintains an oxygen-free environment for the rhizobia to fix N by actively scavenging free oxygen with leghemoglobin carriers (Madsen *et al.*, 2010). This is a critical feature as the bacterially-encoded nitrogenase enzyme utilized for the reduction of N to ammonia is prone to break-down in the presence of oxygen (Dixon and Kahn, 2004).

The epidermal program for bacterial entry and the cortical program for nodule primordium organogenesis converge eventually as the bacteria arrive at and are released into an intracellular space of the dividing nodule primordium cells, where they differentiate and begin fixing N (Fig. 1.6C).

1.6 Genetic elements required for symbiotic signalling

As mentioned previously, both the AM symbiosis and the NFS require perception of chemical stimuli and share a number of signalling elements. For simplicity, the signalling elements required for both the AM symbiosis and NFS will be described based on the extensive knowledge from *L. japonicus*, although many of these loci have also been identified in other model legumes such as *Medicago truncatula* (see Groth *et al.,* 2010 for summation).

Initiation of symbiotic signaling is achieved during the NFS via the activation of two specific NF receptor kinases located at the plasma membrane (NFR1 and NFR5), which are known to bind NF (Radutoiu *et al.,* 2003). These receptors have yet to be identified for the AM symbiosis, but it is likely considering the recently discovered MF that they are similar in appearance to the LysM receptor kinases NFR1 and NFR5. Regardless, extensive genetic analyses conducted on model legumes such as *L. japonicus* have uncovered at least eight shared loci required for both the AM and NFS; thus named the 'common symbiosis genes' (CSG; Fig. 1.7).

The earliest defined participant downstream from NF receptors is the Symbiosis Receptor Kinase or SymRK (Strack *et al.,* 2002), which is thought to relay the initially perceived signal to core downstream elements of the CSG, including three nuclear pore proteins (NUP85, NUP133, and NENA; Groth *et al.,* 2010; Kanamori *et al.,* 2006; Saito *et al.,* 2007). These are believed to act in concert with two gated ion channels CASTOR and POLLUX (Imaizumi-Anraku *et al.* 2005) to produce rapid changes in intracellular calcium concentration (calcium influx and calcium spiking) within the perinuclear and nuclear space. These changes in calcium concentration are thought to be perceived by a

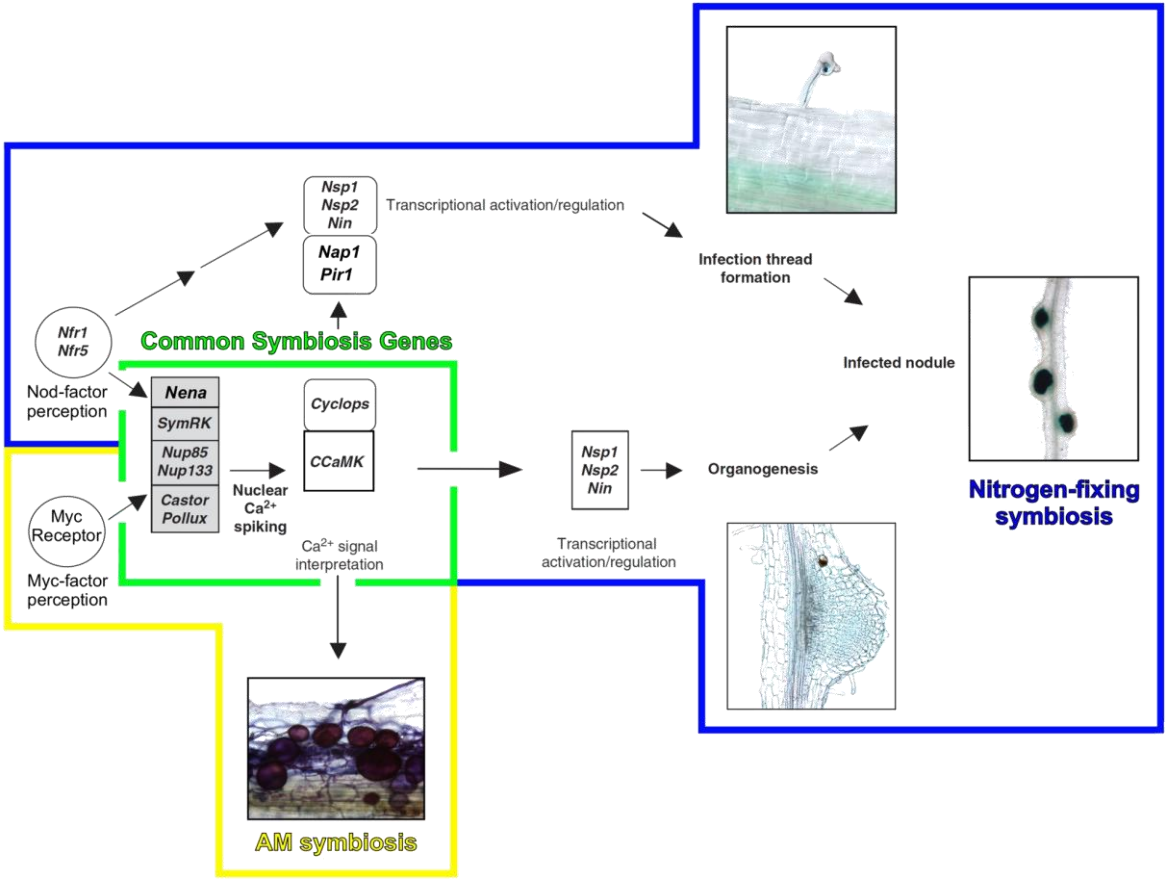


Figure 1.7 Symbiotic signalling elements. Portions of the pathway which are specific to the AM symbiosis are shown in yellow, while those for the NFS are shown in blue. The CSG which are required for both symbioses are outlined in green. For a detailed explanation of genes involved, please see text. Figure modified from Groth *et al.*, (2010).

Ca^{2+}/calmodulin-dependent kinase (CCaMK; Tirichine *et al.,* 2006) and modulated by its' phosphorylation target, CYCLOPS (Yano *et al.,* 2008).

Several, independent mutagenesis screens from model legumes like *L. japonicus*, *Medicago truncatula* and *Pisum sativum* have identified a number of deleterious mutations in all of the aforementioned loci within the CSG, which drastically impair the ability of the plant to form symbiotic interactions with rhizobial or fungal partners (for example, Duc *et al*. 1989, Kistner *et al*. 2005; Amor *et al.,* 2003; Ané *et al.,* 2004; Genre *et al.,* 2002). Downstream of the CSG, signaling pathways for the AM symbiosis and the NFS diverge. Two GRAS-type transcriptional regulators (NSP1/NSP2; Heckmann *et al.,* 2006; Oldroyd and Long, 2003) which serve in a non-redundant fashion, along with the putative transcriptional regulator NIN (for Nodule Inception; Schauser *et al.,* 1999) represent major elements required for bacterial entry and/or nodule organogenesis during the NFS, which are dispensable for the AM symbiosis (Fig. 1.7).

Recently, a number of additional mutants have been described which further dissect this pathway and have revealed yet another set of symbiotic loci which are specifically required for bacterial entry, but not for nodule primordium organogenesis or the AM symbiosis (for recent review see, Held *et al.,* 2010). For example, NAP1 and PIR1 proteins comprise part of the ancient and ubiquitously present SCAR/WAVE complex; a well-characterized complex from mammalian, yeast and plant systems which is responsible for regulating actin polymerization (Goley and Welch, 2006). In a recent breakthrough, the importance of these functions was demonstrated in a symbiotic context from *L. japonicus*, where *nap* and *pir* mutants were able to form nodule structures, yet bacteria did not enter due to deficiencies in IT formation and progression (Yokota *et al.,*

2009, Miyahara *et al.*, 2010). In addition to symbiotic defects, these mutants also display pleiotropic phenotypes including aberrant trichome formation, as well as impaired root and root hair development. The phenotype of these 'entry' mutants has expanded our understanding of the complex genetic network governing symbiotic interactions (Held *et al.*, 2010).

1.7 Cytokinin: the key signalling element for symbiosis

Cytokinins represent a major plant hormone family that are essential for plant growth and development (Heyl and Schmülling, 2003). They were first described by Miller and Skoog (1953), and subsequently utilized by Das *et al.*, (1956) to induce cell division in cultured tobacco pith. They are variable in structure, are all adenine-based derivatives (Mok and Mok, 1994) and are known to be involved in regulating a wide variety of processes such as floral transition (Corbesier *et al.*, 2003), cell-cycle progression (D'Agastino and Kieber, 1999), chlorophyll retention and leaf senescence (Downs *et al.*, 1997), seed development (Emery *et al.*, 1998), as well the regulation of specific organs such as the root and shoot apical meristems (Werner *et al.*, 2003; for review see Perilli *et al.*, 2010).

Extensive research into the signalling elements utilized by these promiscuous hormones has been conducted in model organisms such as *Arabidopsis thaliana* as a means to better understand the molecular mechanisms underlying cytokinin action (Fig. 1.8). Briefly, a wide variety of cytokinin molecules are known to be perceived in the extracellular space by the CHASE (for cyclases/histidine kinases associated sensory extracellular) domain of a well-characterized group of membrane-bound, hybrid histidine kinase (HK) receptors

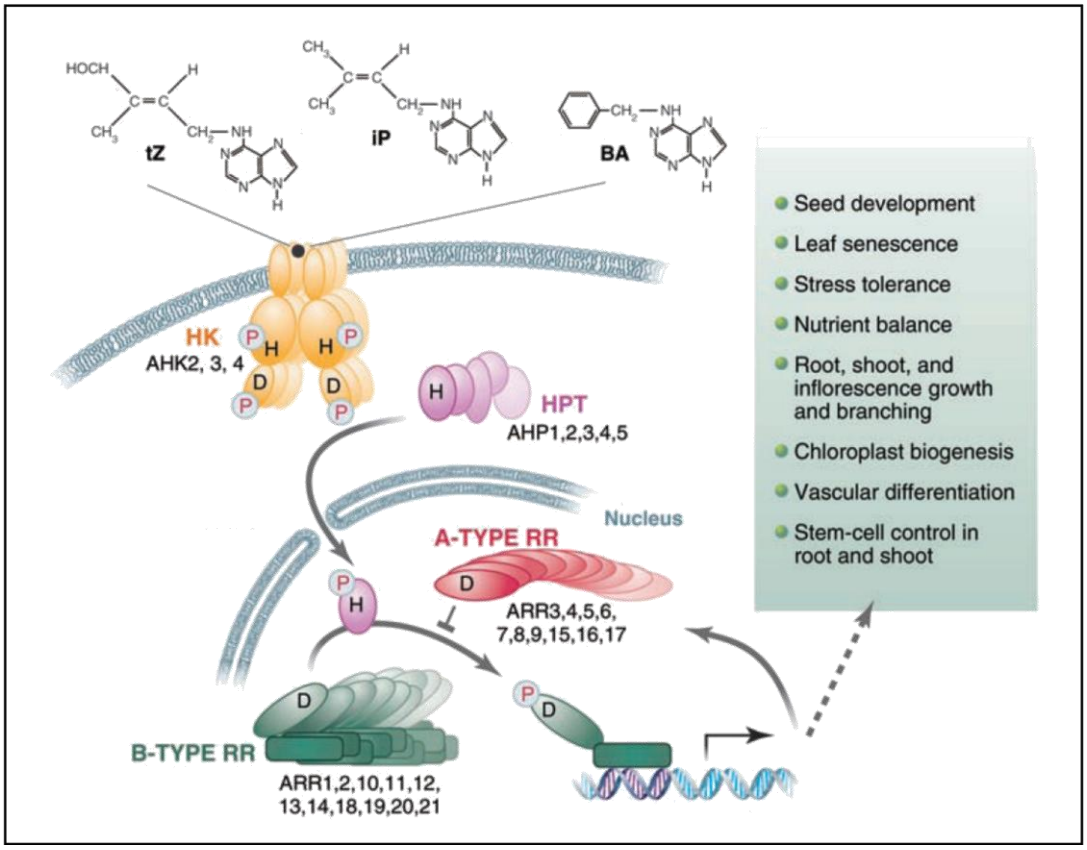


Figure 1.8 Cytokinin signalling elements. The general scheme for cytokinin-induced signal transduction as characterized in *A. thaliana* is shown. tZ, trans-Zeatin; iP, isopentenyl adenine; BA, benzylaminopurine; AHK2/3/4, Arabidopsis Histidine Kinase 2/3/4; H, histidine; D, aspartic acid; P, phosphate; HPT, Histidine Phosphotransfer Protein; RR, response regulator. For detailed explanation, please see associated text. Figure modified from Müller and Sheen (2007).

(Ferreira and Kieber, 2005). Following perception of the ligand and dimerization of the receptor, a phosphorelay is initiated from a conserved histidine residue within the catalytic kinase domain, which is transmitted to a conserved aspartic acid residue in the C-terminal receiver or output domain. Phosphorylation targets of the activated receiver domain include histidine phosphotransfer proteins (HPT), which shuttle phosphate groups into the nucleus, where B-type response regulators (RR) act directly to modulate gene expression of the effecter (Werner and Schmülling, 2009). Negative feedback on the activity of these B-type RRs is achieved by their A-type counterparts (Toh *et al.*, 2007), thus allowing for rapid and specific modulation of cytokinin signalling (Fig. 1.8).

In a symbiotic context, cytokinin turned out to play a critical role in defining the success of mutualistic interactions. It is evident from recent reports that cytokinin signalling is required and also sufficient for nodule primordium organogenesis in at least some legume species. However, we are far from fully understanding the full extent of their involvement in the NFS (Frugier *et al.*, 2008). Since the discovery that cytokinins are produced by a number of symbiotic rhizobia (Sturtevant and Taller, 1989), it has been postulated that these hormones play an important role during the NFS. In agreement with this initial assumption, numerous studies have shown that exogenous application of cytokinin can mimic both NF and rhizobia application by inducing a similar subset of plant cellular responses, such as the activation of cortical cell divisions and accumulation of amyloplasts, as well as transcriptionally activating known early nodulin genes (e.g. *Early Nodulin 40* or *ENOD40* gene expression) in a number of legume systems such as *Trifolium repens*, *L. japonicus*, and *Sesbania rostrata* (Dehio and de Bruijn, 1992; Mathesius *et al.*, 2000; Murray *et al.*, 2007). That cytokinin signalling is indeed tightly

associated with infection has been demonstrated by analysing expression of the *proARR5::GUS* fusions (ARR5 is a cytokinin response regulator in *A. thaliana*; Lohar *et al.,* 2004). The *ARR5* promoter (*proARR5*) was shown to be rapidly activated, as measured by the accumulation of the GUS reporter activity, upon rhizobial inoculation both in the root epidermis and in the root cortex in association with nodule primordium organogenesis (Lohar *et al.,* 2004).

Furthermore, study of alfalfa (*Medicago sativa*) root nodules induced by *Sinorhizobium meliloti* produced a number of bacterial mutants unable to induce nodule formation, some of which contained mutations in the canonical *nodABC* genes, responsible for NF production (Cooper and Long, 1994). When the *Agrobacterium tumefaciens* secretion system (pTZS), which constitutively produces cytokinins, was transformed to the *S. meliloti* non-nodulating (Nod^-) strain that was unable to synthesize NF, this restored the ability of the mutant bacteria to induce the formation of nodule structures on *M. sativa* roots (Cooper and Long, 1994). All these reports clearly indicated that cytokinin plays a pivotal role during the NFS. However, in spite of all these observations, the direct evidence which points to the necessity for rhizobia-derived cytokinins during signalling for NFS has never been obtained (Frugier *et al.,* 2008).

What has become clear, however, is that the cytokinin signalling, likely originating as a response of the plant host to bacterial inoculation, acts downstream from NF perception to mediate the cortical program for nodule primordium inceptions (Gonzalez-Rizzo *et al.,* 2006, Murray *et al.,* 2007, Tirichine *et al.,* 2007).

In *L. japonicus*, cytokinin signalling through LHK1 (LOTUS HISTIDINE KINASE 1) cytokinin receptor is not only required, but also sufficient for nodule

organogenesis (Murray *et al.,* 2007; Tirichine *et al.,* 2007). *L. japonicus* plants carrying loss-of-function mutations in *Lhk1* fail to produce timely cortical cell divisions in spite of the fact that these mutants were hyperinfected by rhizobia (Murray *et al.,* 2007). Moreover, a gain-of-function mutant of *Lhk1,* called *spontaneous nodule formation 2* or *snf2,* was identified, which formed empty nodules (i.e. nodules without bacteria) in the absence of rhizobia (Tirichine *et al.,* 2007), thus solidifying the proposal that cytokinin signalling through *Lhk1* is required and also sufficient for nodule organogenesis in the root cortex.

From the detailed analyses in *L. japonicus*, we know that cytokinin signalling is tightly linked to the regulation of NIN, a transcription factor which plays dual role in two spatially separated positions (Radutoiu *et al.,* 2003). Upon activation by the LHK1-dependent cytokinin signalling, NIN is required in the root cortex for the inception of nodule primordia. In the root epidermis, NIN appears to have a complex role. It initially supports bacterial infection and colonization (Schauser *et al.,* 1999). However, once infection occurs and nodule organogenesis is initiated, NIN also regulates the susceptibility of the root by restricting further colonization by the bacteria (Radutoiu *et al.,* 2003; Tirichine *et al.,* 2006). Signalling through LHK1 is required for the activation of NIN in the cortex and therefore nodule organogenesis, as *lhk1-1* mutants, which have dysfunctional cortical program, showed a reduced expression of NIN upon cytokinin application (Murray *et al.,* 2007).

Although induction of cytokinin signalling in root epidermis upon bacterial inoculation was demonstrated (Lohar *et al.,* 2004), the infection process is LHK1 independent (*lhk1* loss-of function plants are hyperinfected ; Murray *et al.,* 2007) and the

role, if any, of cytokinin in mediating root colonization by bacteria remains rather controversial (Frugier *et al.,* 2008)

1.8 Goal of the thesis

Considering that only a very limited number of flowering plants, mostly legumes, form nodules, has the evolution of nodulating plants entailed novelty in cytokinin signaling? Cytokinin receptors and signalling elements are ubiquitously present in all plants, so what dictates their specialized role in leguminous plants?

Although cytokinins clearly play a critical role during N fixing symbiosis (see Section 1.7), many questions remain unanswered. For example, how does NF perception at the root surface link with the inherent plant hormonal machinery and what is the nature of cytokinin signaling during symbiosis; does de-novo cytokinin biosynthesis occur or is it perhaps translocation of cytokinin from other parts of the plant that matters? Furthermore, is cytokinin signaling in addition to its role in nodule organogenesis also required for bacterial entry into the root, as recently discussed (Frugier *at al.,* 2008)?

Although the LHK1 cytokinin receptor has been defined as required and also sufficient for the nodule organogenesis (Murray *et al.,* 2007; Tirichine *et al.,* 2007), mutant plants carrying *lhk1* loss-of–function alleles still form a limited number of aberrant nodules (Murray *et al.,* 2007). Is this rudimentary nodulation reflective of a cytokinin-independent signaling process or the result of at least partial functional redundancy among cytokinin receptors? Thus, is LHK1 function unique during nodulation (among cytokinin receptors in *L. japonicus*) and if indeed it is, what attributes of the gene and/or protein define this phenomenon?

Answering these and related question should allow for an important insight into the evolution of this fascinating plant developmental process. Moreover, the knowledge gained from pursuing this topic should bring us closer to the prospect of a rational evaluation of the potential for engineering nodule primordium organogenesis in currently non-nodulating plants.

The goal of this thesis therefore, was to begin addressing some of these key questions. Ground-breaking experiments are reported herein, which identify and functionally characterize the cytokinin receptor gene family in a model legume *L. japonicus* in the context of its role in the establishment of functional NFS. The central hypothesis is that the role of LHK1 during the NFS is not unique - other members of this receptor family act redundantly to LHK1 at the root epidermis to promote bacterial colonization and therefore allow for the persistence of infection and sparse nodule organogenesis observed in *lhk1-1* mutants. The following specific objectives were proposed:

1. To characterize the abundance, genomic context, and protein domain structure of histidine kinase cytokinin receptors in the *L. japonicus* genome.

2. To assess the cytokinin-responsive function of putative LHK receptors (*Lhk1A*, *Lhk2*, and *Lhk3*) using heterologous systems in yeast and *E. coli*.

3. To create mutant lines for all newly described *Lhk* loci via reverse genetics (TILLING) and assess the effect of these presumed, deleterious mutations on *Lhk1A*, *Lhk2*, and *Lhk3* gene function via heterologous systems as above.

4. To analyze the effect mutations in *Lhk1A*, *Lhk2*, and *Lhk3* on the development of the NFS through extensive phenotypic characterization.

5. To determine the spatial/temporal expression pattern for *Lhk* gene promoters using reporter constructs transformed into stable transgenic lines.

6. To interrogate the biochemical interchangeability of different LHK receptors by using *in planta* complementation of the *lhk1-1* mutant.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant growth conditions

Seeds of *L. japonicus* were scarified lightly prior to surface sterilization. A solution of 70% ethanol and 0.1% sodium dodecyl sulphate (SDS) was used for initial sterilization, followed by one rinse with sterile Milli-Q^{TM} water and treatment with a solution containing 20% bleach and 0.1% SDS. Sterilized seeds were then rinsed ten times with sterile water and allowed to imbibe overnight. After imbibition, germination was conducted in standard 100 x 15mm Petri plates sealed with Parafilm and containing 6-8 layers of sterilized Whatmann filter paper (Fischer #09-801A), moistened with sterile Milli-Q H_2O. Seedlings were routinely germinated for a period of 7 days under continuous light at 23^{o}C.

2.2 Analyses of symbiotic phenotypes

For nodulation assays, seedlings were transferred (under sterile conditions) to pots containing a mixture of vermiculite and coarse sand (6:1) and watered with a 1X B & D nutrient solution, containing 0.5mM KNO_3 (Broughton and Dilworth, 1971). Growth conditions were dictated by a light (18hr ON, 6hr OFF) and temperature (23^{o}C, 18^{o}C) regime. After 7 days of growth, these seedlings were inoculated with either a wild-type *Mesorhizobium loti* strain (NZP 2355), or *M. loti* containing the *hemA::lacZ* cassette for visualisation of bacterial infection via a standardized β-galactosidase staining procedure (Wopereis *et al.* 2000). This system was employed for the analysis of early infection events, generally scored at 7 days after inoculation (7 DAI) as well as later events scored at 14 and 21 DAI.

For mycorrhizal assays, seedlings were transferred to pots containing a mixture of Turface™ (MVP) and silica sand (1:1) and watered with ½ strength Hoagland's nutrient solution lacking phosphate (Hoagland and Arnon, 1950). Inoculation was achieved via a mixture of cultured leek and chive roots, as well as application of a commercial inoculation containing a mixture of *Glomus spp.* fungi (MykePro™, Premier Tech Biotechnologies). Plants were routinely harvested eight weeks after inoculation and stained for mycorrhizal colonization using a standard, ink-vinegar protocol (Vierheilig *et al.*, 1998). Selected roots were cleared for 1hr in 10% KOH at 75°C, followed by one rinse with sterile ddH_2O. Staining proceeded for 1hr at room temperature in 5% ink (Schaeffer black)-vinegar (5% acetic acid). Lastly, the roots were rinsed once more with sterile ddH_2O and de-stained in 5% acetic acid for 20min before being stored at 4°C in ddH_2O.

2.3 Identification of putative cytokinin receptors in *L. japonicus*

TAC sequences containing putative *L. japonicus* HK cytokinin receptors isolated from the *L. japonicus* genome were kindly provided by Dr. Shuei Sato (Kasusza DNA Research Institute, Japan). Exon/intron structure of all loci was predicted using the GenScan server (http://genes.mit.edu/GENSCAN.html) and validated using standard PCR and RT-PCR techniques. The CLUTSALW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and BoxShade (www.mobyle.pasteur.fr/cgi-bin/portal.py?form=boxshade) software programs were used for the protein/DNA alignments, whereas TreeView Win32 (www.taxnomoy.zoology.gla.ac.uk/rod/rod.html)

was used for the development of phylogenetic trees (bootstrap values calculated 1000 times).

2.4 *RACE* validation and expression of putative *Lhk* ORFs in *L. japonicus*

Total mRNA was isolated from *L. japonicus* (Gifu) nodules with the RNeasy Plant Mini kit (Qiagen) and treated with DNAseI. Full length cDNAs for *Lhk1A, Lhk2,* and *Lhk3* were generated by 5' and 3' Rapid Amplification of cDNA Ends (RACE) using the FirstChoice RLM-RACE kit (Ambion). For semi-quantiative reverse transcriptase polymerase chain reaction (RT-PCR), cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen) from uninoculated root tissue, nodules and leaves of wild-type Gifu plants. *Lhk* transcripts were routinely amplified from these tissues using High Fidelity Platinum Taq (Introgen) and subsequently sequenced. The PCR program was as follows: 94^{o}C 5min, followed by 30 cycles of 94^{o}C 30sec, 58^{o}C 30sec, 68^{o}C 30sec, with a final extension for 7min at 68^{o}C. Gene-specific primers designed to amplify products encompassing the final exon and 3' UTR were as follows:

(*Lhk1A*)	F 5'-ATGGACGGATTTGAAGCAAC-3'
	R 5'-CAAGATCTCTTTCGGTCTGC-3'
(*Lhk2*)	F 5'-CACTCATTGCAGGAGAAGAGG-3'
	R 5'-TTTTCCATCTTAGCCCCTCA-3'
(*Lhk3*)	F 5'-TGGAACACAATGTGAACAGAGA-3'

R 5’-CCCATTTCTCCCATCCTTCT-3’

Lhk1 RT-PCR primers were to those used in Murray *et al.*, (2007).

2.5 Alternative splicing at the *Lhk3* locus

Primers were designed for PCR-based detection- and expression-based studies of the two, alternative splice variants produced from the *Lhk3* locus (named *Lhk3* variant #1 and variant #2). For the comparative analysis of these two, alternatively-spliced transcripts, a 5’ product was amplified from cDNA template of various tissues using a common reverse primer and a transcript-specific, forward primer. The PCR program was as follows: 5 min denature at 94°C, followed by 30 cycles of 94°C 30 sec, 58°C 30 sec, 68°C 1 min, followed by a 7 min soak at 68°C.

The primers are as follows:

(*Lhk3* variant #1) F 5’-CTTATATGAAGGGTGGTTTTGG-3’

R 5’- CTTTCCAGAAAGCACGTCAAC -3’

(*Lhk3* variant #2) F 5’- GGTTGGTTACTGTTGTGGATGA -3’

R 5’- CTTTCCAGAAAGCACGTCAAC -3’

2.6 Isolation of *Lhk* mutants

Targeted Induced Localized Lesions IN Genomes (TILLING; Perry *et al.*, 2003) was utilized for the isolation of mutants in the *Lhk1A*, *Lhk2*, and *Lhk3* loci. The

TILLING approach was limited to a 1kb region encompassing mainly a single, large exon of the highly conserved and functionally required kinase domain based on predictions made by the CODDLE software (http://www.proweb.org/coddle/). This reverse genetics approach produced a number of point mutations in each locus analyzed. Mutations falling outside the coding region (i.e. within introns), or those that created synonymous and conservative substitutions were largely disregarded. All selected lines were immediately back-crossed before extensive phenotypic analyses were conducted to ensure that no additional mutations skewed the data.

For the creation of double mutant backgrounds, genetic crosses were conducted between homozygous single mutants for each of the two loci being analyzed. The F1 plant was allowed to self-fertilize and produce the F2 segregating population, where the desired double mutant was selected for using a combination of sequence analysis, cleavage amplification polymorphisms (CAPS), and derived CAPS (dCAPS) markers depending on the line. Only F3 progeny from confirmed, homozygous double mutants was utilized for phenotypic evaluation.

2.7 Cytokinin-responsive assay in *Saccharomyces cerevisiae*

All *Lhk* cDNAs were directionally cloned into the multi-cloning site of a yeast expression vector (P415CYC; Mumberg *et al.* 1995) as follows:

Lhk1A - *HindIII* and *SalI*

Lhk2 - *EcoRI* and *SalI*

Lhk3 splice variant #1 - *SpeI* and *HindIII*

Lhk3 splice variant #2 - *SpeI* and *HindIII*

The previously analyzed *Lhk1* cDNA was used as a positive control (Murray *et al.*, 2007). Subsequent constructs were transformed into the *sln1Δ* mutant of *Saccharomyces cerevisiae* (kind gift from Tatsuo Kakimoto, Osaka University, Japan) and analyzed for response to exogenous treatment with different plant hormones including specific ligands such as benzyl-amino purine (BAP) and trans-zeatin (tZ), as well non-active cytokinin forms such as cis-zeatin (cZ) and the non-specific ligand 1-Naphthaleneacetic acid (NAA; as in Murray *et al.*, 2007). Suspensions of transformations were spotted out onto –LEU –URA drop-out media, with or without 2% galactose (GAL), in the presence or absence of the aforementioned hormones at a final concentration of 10μM. Putative loss-of-function mutations were also interrogated using this assay.

2.8 Cytokinin-responsive assay in *Escherichia coli*

The appropriate *Lhk* cDNAs were cloned into the *E. coli* expression vector *pSTV28* as follows:

Lhk1 – *SacI* and *SalI*

Lhk2 - *EcoRI* and *SalI*

Lhk3 splice variant #1 - Sac*I* and *SalI*

Lhk3 splice variant #2 - Sac*I* and *SalI*

Subsequent constructs were transformed into the sensor-negative *SRC122 E. coli* strain (kind gift from Dr. Takafumi Yamashino; Japan). Following transformation, colonies were grown on LB plates containing 40mM sodium phosphate buffer and 20mM glucose, with or without the addition of 200μM BAP for two days at 25^{o}C (Miwa *et al.*, 2007). Activated blue colonies due to the engineered *cps::lacZ* fusion were clearly

visible from the control. This same media preparation was again employed for liquid cultures destined for analysis of β-galactosidase activity using a standard assay as described in Tirichine *et al.*, (2007).

Putative loss-of-function mutations were also interrogated using this assay. The Quikchange XL II Site-directed mutagenesis kit (Stratagene) was used for the creation of *Lhk* loss-of-function constructs according to the manufacturer's protocol.

2.9 Root elongation and cytokinin insensitivity assays

The extent of root growth was assessed for various genotypes as indicated in the text using standard protocols as described (Wopereis *et al.*, 2000). After two days of germination upside-down on standard Petri plates (Section 2.1), exposed radicles were carefully transferred to vertical plates containing ½ B5 with minimal organics, 2.5mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 4.5% sucrose, and 0.8% phytagel. Roots were allowed to elongate for seven days at 23^{o}C. The average extent of elongation was scored for 15-20 roots per genotype.

For hormone treatments, the growth conditions and media were identical to those described above. Where appropriate, BAP was added (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M). The control plates received no hormone treatment.

2.10 Production of stable transgenics

To create promoter fusions for *Lhk1*, *Lhk1A*, *Lhk2*, and *Lhk3*, a 4kB fragment upstream of the 5' untranslated region (UTR) sequence was first isolated, amplified and cloned using Gateway^{TM} technology (Invitrogen) into the promoterless *pKGWFS7,0*

destination vector (GUS and GFP reporters; t35S terminator sequence). After validation of the insert using sequence and restriction digest, the corresponding vectors were transferred to *Agrobacterium tumefaciens* LBA4404 using electorporation. Standard transformation protocols were used to regenerate full transgenics from hypocotyl segments of wild-type (ecotype '*Gifu'*) plants. No less than ten, independent transformants were isolated for analyses of promoter expression, following confirmation of transformation by PCR screen and GUS staining.

2.11 GUS staining procedures

Various tissues were isolated from transgenic plants, including nodules, roots, and also whole seedlings at the timepoint indicated in the text for histochemical staining. Detection of the GUS reporter activity was routinely conducted using a staining solution which contained 0.1M potassium phosphate buffer, 5mM EDTA, 0.5mM potassium ferric- and ferrous-cyanides, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide cyclohexylammonium salt (X-GLUC; Fermentas). Triton X-100 (Sigma; 0.1% v/v) was added for leaf staining due to the hydrophobic cuticle of *L. japonicus*. All tissues were vacuum-infiltrated for 15min, stained overnight at room temperature and cleared as described previously (Wopereis *et al.*, 2000).

2.12 Hairy-root complementation of *lhk1-1* mutants

2.12.1 Over-expression of *Lhk* cDNAs

To over-express different *Lhk* cDNAs in the *lhk1-1* mutant background, *Lhk* cDNAs were cloned into the *pEarleygate100* destination vector (35S promoter, 3'

octapine synthase (OCS) terminator) using the Gateway™ technology and subsequently transformed into *A. rhizogenes* strain AR10, as describe above for *A. tumefaciens* transformation. *A. rhizogenes*-mediated transformation procedures were followed to induce the formation of hairy roots from *lhk1-1* mutant seedlings (Petit *et al.*, 1987). The inoculated plants containing hairy roots were transferred to soil and assessed for nodulation at 21 DAI as described above (see section 2.2). No less than 10 plants were scored for each genotype, per experiment.

2.13 Microscopy and image analysis

All microscopic observations were performed on a Nikon SMZ1500 (Nikon, Japan) dissecting or the Zeiss Axioscope 2 (Zeiss, Germany) compound light microscope. Both microscopes were integrated with a Nikon DXM1200 digital camera using the ACT1 media software (Nikon). Optical ranges for the Nikon dissecting scope varied between 0.75 and 11.25X, while the compound Zeiss microscope was fitted with 10, 20, 40, and 63X objectives. All images captured were taken in a TIFF format at a resolution of 3840 x 3072.

2.14 Statistical analyses

In all cases, means were calculated from data ranges containing no less than 10 plants per genotype, per treatment. All data were conformed to a normal distribution as confirmed by the Pearson's test and subsequent pair-wise comparisons were made using a Student's t-Test assuming unequal variance. Unless otherwise stated, variance shown in all cases represents the 95% confidence intervals of the mean.

2.15 BLAST analyses

The Blast Local Alignment Sequence Tool (BLASTp) was used for comparison of predicted LHK protein sequences using the default parameters provided at http://blast.ncbi.nlm.nih.gov. A maximum of 100 matches were analysed, with a minimum word size of 3 and maximum matches to a query range set to zero. The matrix utilized was BLOSUM62 since the query sequences were generally long (>900 amino acids) and provided strong matches to sequences within the database. Gap costs were set to 11 and 1 for existence and extension, respectively with compositional adjustments set to a conditional compositional score matrix.

CHAPTER 3

RESULTS

3.1 Disclaimer and co-authorship

Dr. Jillian Perry and Dr. Trevor Wang (John Innes Centre, Norwich, UK) conducted the identification of mutations for all *Lhk* loci via the TILLING process.

Christian Huynh participated in the cloning of *Lhk* cDNAs for complementation and functional analysis.

Dr. Hungwei Hou participated in the cloning of the *Lhk2* cDNA for functional analysis.

Dr. Krzysztof Szczyglowski provided research direction and critically reviewed all material covered in the thesis.

3.2 The *L. japonicus* cytokinin receptor family is comprised of four members

Recent advances in generating the *L. japonicus* whole-genome sequence (Sato *et al.*, 2008; see also Szczyglowski and Stougaard, 2008) made it possible to comprehensively investigate the organization of the *L. japonicus* histidine kinase (*Lhk*) cytokinin receptor gene family. Using the Blast server (www.blast.ncbi.nlm.nih.gov/Blast), available genomic DNA sequences, and the predicted protein sequences of the previously described *Lhk1* gene and the partial sequence of the *Lhk2* cDNA (Murray *et al.*, 2007) as queries, two additional putative members of the *L. japonicus* cytokinin gene family were identified. As well, the complete genomic sequence of *Lhk2* was obtained (see below).

Based on overall sequence homology and the clustering pattern of LHK receptors with predicted orthologues in *A. thaliana*, *M. truncatula*, and *P. sativum* (see below), *Lhk2* was renamed to *Lhk1A*, while the two newly identified putative cytokinin receptors genes were named *Lotus histidine kinase 2* (*Lhk2*) and *Lotus histidine kinase 3* (*Lhk3*), respectively. The names for the corresponding predicted proteins were designated as LHK1 (Lotus Histidine Kinase 1), LHK1A, LHK2 and LHK3.

3.3 Genomic organization of *Lhk* loci

All four *Lhk* genes were positioned onto the *L. japonicus* genetic map using available genomic sequence information (Sato *et al.*, 2008). The *Lhk1* and *Lhk3* loci were localized to the large genomic clones, TM1769 and TM2189, positioned at the lower and upper arm of chromosome 4, while *Lhk1A* and *Lhk2* were found on the upper and lower arm of chromosome 2, as represented by BM2030 and TM2188, respectively (Fig. 3.1A).

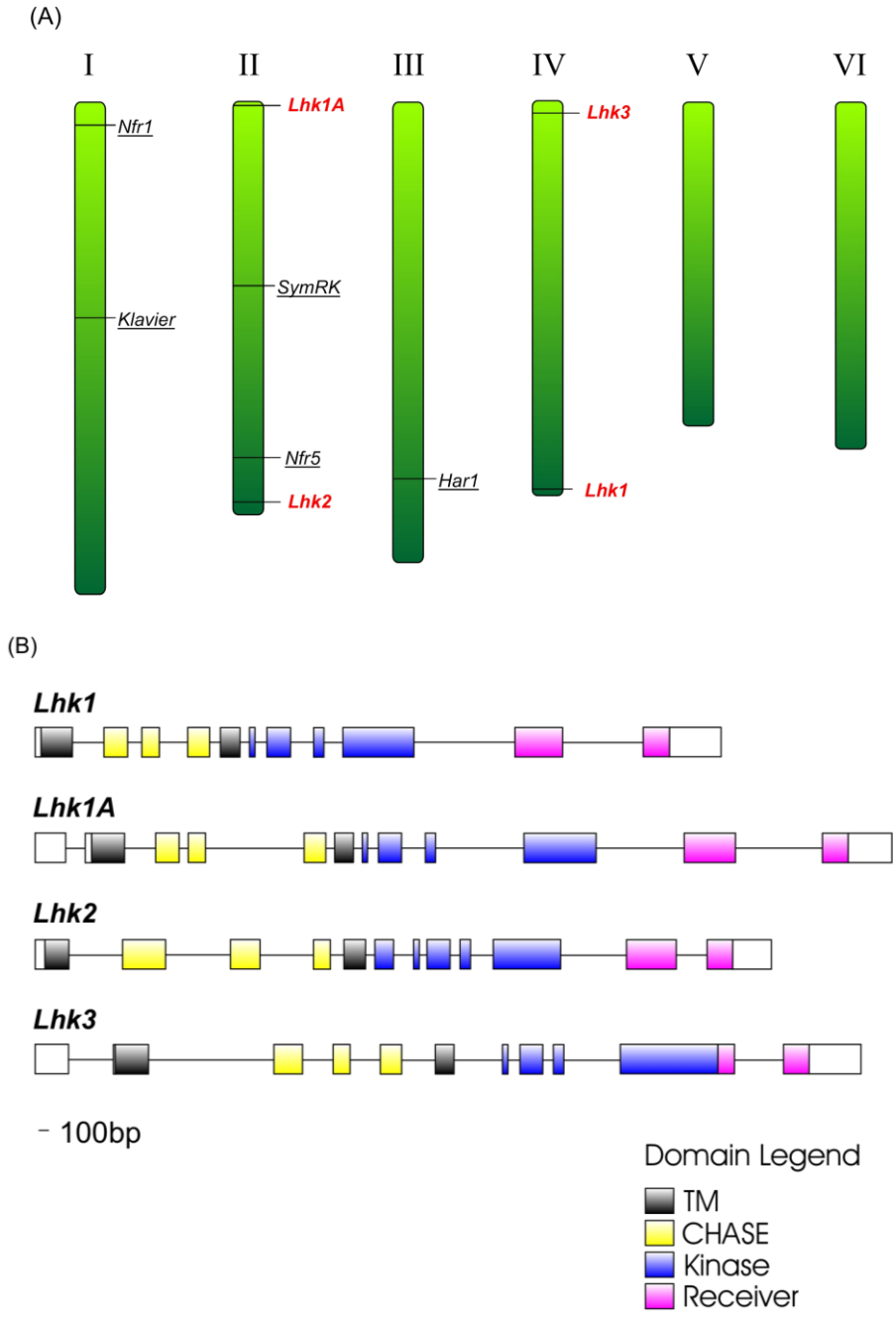


Figure 3.1 Genomic context of *Lhk* loci. (A) The genetic map of *L. japonicus* is shown, highlighting the location of *Lhk* loci along with other known receptor kinases such as the NF receptors (*Nfr1*, *Nfr5*), the receptor-like kinase *SymRK* and two kinases which are involved in the regulation of nodule number *Har1* and *Klavier*. (B) Predicted exon/intron structure of *Lhk* genes. Boxes represent exons, while lines represent introns. The 5' and 3' UTRs are shown as open boxes. The protein domain that a given exon is predicted to contribute to in the translational product is color-coded as per the legend. TM- transmembrane domain; CHASE: predicted ligand (cytokinin)-binding extracellular domain; Kinase: conserved protein kinase domain; Receiver: conserved C-terminal output domain.

Full-length transcripts were obtained for the three newly described *Lhk* genes using the 5'- and 3'-RACE. Subsequently, a variety of on-line tools, such as GenScan (www.genes.mit.edu/GENSCAN.html) and Spidey (www.ncbi.nlm.nih.gov/spidey) were utilized to determine the exon/intron structure for these loci (Fig. 3.1B).

Divergence of intron length among different *Lhk* genes was noticed (Fig. 3.1B). However, the number and size of major exons was conserved amongst different *Lhk*s; the *Lhk1* and *Lhk3* genes had 11 exons, there were 12 predicted exons representing both the *Lhk1A* and *Lhk2* loci. The length of the predicted open reading frames was found to be 2994 bp, 3657 bp and 2961 bp for *Lhk1A*, *Lhk2*, and *Lhk3* respectively. These reflected the corresponding proteins of 997, 1219 and 987 amino acids in length, with predicted molecular masses of 111, 136, and 110 kDa for LHK1A, LHK2, and LHK3, respectively. When the predicted translational products were superimposed on the corresponding gene structures, their topology was found to be rather conserved amongst different *Lhk*s (Fig. 3.1B).

3.4 Phylogeny of LHK receptors

Alignments of all four predicted *L. japonicus* LHK receptors to other known cytokinin receptors from legumes such as *M. truncatula* and *Pisum sativum* as well as from a non-legume, *A. thaliana* positioned these proteins into three distinct groups (Fig. 3.2). The first group consisted of LHK1 and LHK1A and their presumed counterparts, *A. thaliana* Histidine Kinase 4 (AHK4), *M. truncatula* Histidine Kinase 1 (MtHK1), and *P. sativum* Histidine Kinase 1 (PsHK1). The second group encompassed LHK2, AHK2 and MtHK2, while the third group contained proteins most similar to LHK3 (Fig. 3.2).

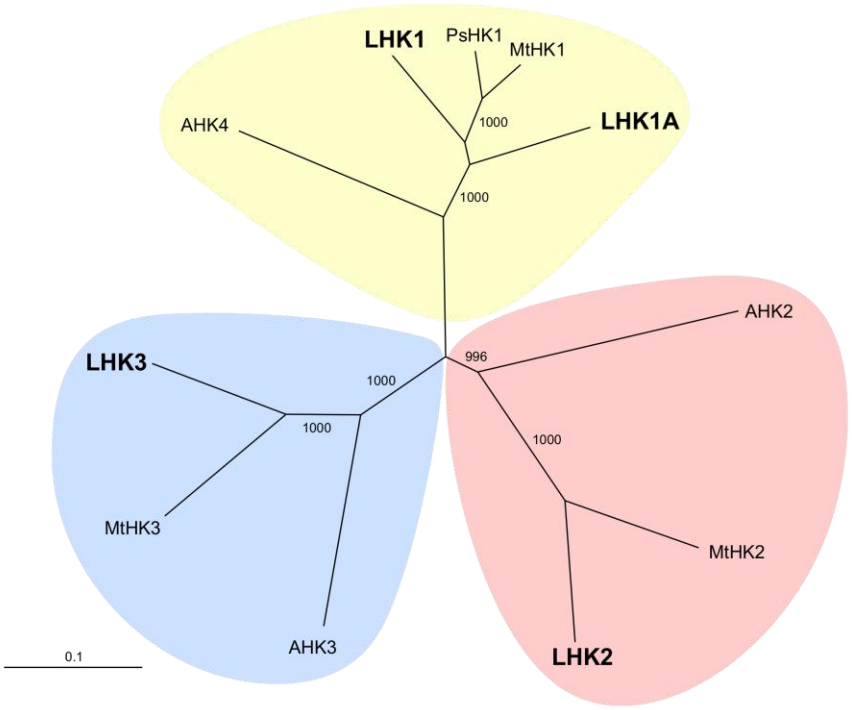


Figure 3.2 Phylogeny of the *Lotus japonicus* LHK proteins. The unrooted tree is based on an amino acid alignment of full-length sequences from *L. japonicus* (LHK; Genbank accession numbers: LHK1, DQ848999; LHK1A, DQ848998; LHK2, this work; LHK3, AP009230; *A. thaliana* (AHK4, NP_565277.1; AHK2, NP_568532.1; AHK3, NP_564276.1), *M. truncatula* (MtHK1, ABE94286; MtHK2, CT571263X; TIGR TC accession number: MtHK3, TC105528), and *P. sativum* (PsHK1, DQ845485). Protein sequences were aligned with CLASTALW2 using the default settings. The phylogenic tree was created in TreeView v32, with bootstrap values and alignments calculated 1000 times.

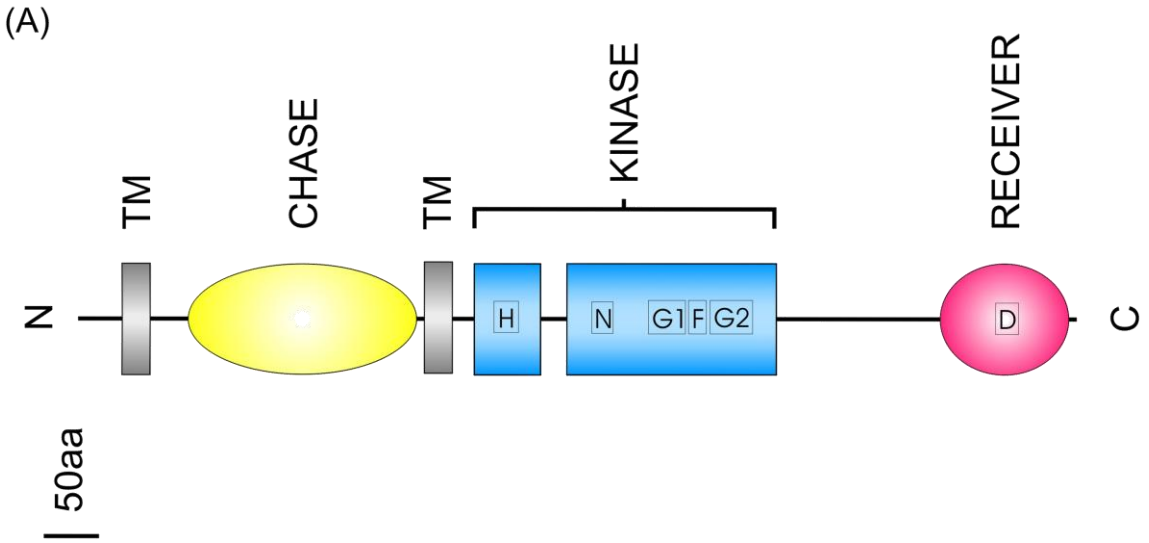
The similarity of LHK protein sequences within the predicted *L. japonicus* cytokinin receptor family and to representatives from *A. thaliana* is summarized in Table 3.1. LHK1 and LHK1A were the most similar, sharing 80% identity at the amino acid level. Conservation between these two proteins and the AHK4 cytokinin receptor was also high at 67 and 69%, respectively. In contrast, LHK1 and LHK1A shared only ~50% identity with LHK2 and LHK3. Amino acid sequence conservation was greater in between both LHK2 and LHK3 and also to their presumed orthologues from *A. thaliana* (AHK2 and AHK3) than between these two proteins and LHK1 or LHK1A (Table 1).

3.5 LHK proteins contain characteristic domains of known cytokinin receptors

To further investigate the prediction that the LHK proteins indeed constitute functional cytokinin receptors, their amino acid sequences were analysed for the presence of protein domains (Fig. 3.3A). The site of action for the cytokinin receptors has classically been predicted to be the plasma membrane and accordingly, the WoLF PSORT software (http://wolfpsort.org/) heavily favoured localization to the plasma membrane for all LHKs, with the derived probability for LHK1, LHK1A, LHK2, and LHK3 being 0.92, 0.92, 0.69, and 0.92, respectively (on a scale of 0 - 1). The N-terminal portion of all LHKs was found to contain a predicted extracellular CHASE domain, which is thought to be anchored to the plasma membrane by flanking transmembrane (TM) domains (Schmülling, 2001). Although similar in structure to extracellular perception apparati in prokaryotes and lower eukaryotes (Stock *et al.*, 2000), the CHASE domain is unique to cytokinin receptors in higher plants (Schmülling, 2001) and was found to be highly conserved within the LHK family and also between LHKs and CHASE domains of other known cytokinin receptors, such as AHK4 (Fig. 3.3B). As

Table 3.1 Amino acid conservation of LHK homologues and presumed AHK counterparts.

Name	Length	Name	Length	Identity (%)	Similarity (%)
LHK1	993	LHK1A	997	80	86
LHK1	993	LHK2	1218	52	67
LHK1	993	LHK3	986	49	66
LHK1	993	AHK4	1080	67	78
LHK1A	997	LHK2	1218	53	68
LHK1A	997	LHK3	986	51	68
LHK1A	997	AHK4	1080	69	79
LHK2	1218	LHK3	986	54	69
LHK2	1218	AHK2	1176	59	71
LHK3	986	AHK3	1036	68	80



(B)

```
LHK1   NTSAIDQETFAEYTARTAFERPLMSGVAYAQRVVHSERERFEKQHGWVIKTMER------      54
LHK1A  NPSAIDEETFAEYTARTAFERPLLSGVAYAQRVINSERERFEKQHGWVIKTMER------      54
AHK4   NPSAIDQETFAEYTARTAFERPLLSGVAYAEKVVNFEREMFERQHNWVIKTMDRG-----      55
LHK2   NPSAVDQKIFGEYTESTAFERPLTSGVAYAFKVLHSERIHFENQHGWTIKKMESENE--A      58
LHK3   NPSTIDQRTFARYTDRTAFERPLTSGVAYAVRVLQSEREQFEKQQGWTIKRMDTVEQNPV      60

LHK1   ----------VPSGVRDEYAAVIFAQETVSYLESIDMMSGEEDRENILRARATGKAVLTS      104
LHK1A  ----------ESSQVRDEYAPVIFAQETVSYLESLDMMSGEEDQENILRARATGKAVLTS      104
AHK4   ----------EPSPVRDEYAPVIFSQDSVSYLESLDMMSGEEDRENILRARETGKAVLTS      105
LHK2   LVQDCIPENLDPAPIQDEYAPVIFAQETVSHIVSIDMMSGKEDRENILRARASGKGVLTS      118
LHK3   HKDDYAPEKLEPSPIQEEYAPVIFAQDTVSHVISVDVLSGKEDRGNVLRARESGKAVLTA      120

LHK1   PFRLLDSHHLGVVLTFPVYKSKLPPEPTTEEVIKAIAGYIGGSFDVESLVENLLGQLAGN      164
LHK1A  PFRLLGSHHLGVVLTFPVYKSKLPPKPTLEERIEATAGYVGGSFDVESLVENLLGQLAGN      164
AHK4   PFRLLETHHLGVVLTFPVYKSSLPENPTVEERIAATAGYLGGAFDVESLVENLLGQLAGN      165
LHK2   PFKLLKSNHLGVVLTFAVYNSKLPPDATVEQRVEATVGYLGASYDIPSLVDKLLHQLASK      178
LHK3   PFRLLKTNRLGVILTFAVYKKDLPSNATPNERIQATDGYLGGVFHVESLVDKLLQQLASK      180

LHK1   QAILVKVYDITNSSDPLIMYGSQYEEGDMSLVHESKLDFGDPYRKHHMI     213
LHK1A  QAILVNVYDITNCSNPLTMYGNHSEEADMSLSYESKLDFGDPYRIHQMI     213
AHK4   QAIVVHVYDITNASDPLVMYGNQDEEADRSLSHESKLDFGDPFRKHKMI     214
LHK2   QTIVVNVYDTTNASSPIAMYG--TDVADTGLLKIRSIDFGDPLRKHEMH     225
LHK3   QTVNVDVYDTTNHTHPIAMYG--SNVSRDAFYHVSSLNFGDPFRKHEMH     227
```

Figure 3.3 (A). A generalized structure of LHK cytokinin receptors. Transmembrane (TM) domains are shown flanking the extracellular CHASE domain where the ligand is perceived. The phosphorylation of conserved histidine residue within the cytosolic kinase domain initiates a phosphorelay, which is transferred to the C-terminal receiver or output domain. H, N, G1, F, and G2, box motifs of the kinase domain are highlighted as well as a conserved aspartic acid within the receiver domain (D). (B) Alignment of the CHASE domain from all LHK receptors with the well-characterized CHASE domain of AHK4. The alignment was created using CLUSTALW2 and analyzed by the BoxShade software. A threshold of ≥80% conservation was used. Green shading indicates identical residues, whereas yellow indicates conservative substitutions.

expected, the predicted cytosolic portion of the LHK proteins was found to be largely comprised of the highly conserved kinase domain. Found within this extensive catalytic domain were the canonical H, N, G1, F, and G2 box motifs known to be functionally required (Parkinson and Kofoid, 1992), as well as a highly conserved histidine residue at the N-terminal end, known to participate in signal transduction (Fig. 3.4). Downstream, a C-terminal receiver or output domain was also identified in all LHK receptors. This domain is known to participate in the phosphotransfer from the kinase domain to downstream signalling elements, such as HPT proteins (Ferreira and Kieber, 2005). The receiver domain carries three, well-defined and characteristic motifs named the DD, D, and K motifs for the conserved residues found within them (Ueguchi *et al.,* 2001). All such conserved motifs were observed in putative LHK receptors, including an absolutely conserved aspartic acid in the D motif, known to transfer phosphate groups to trans-acting HPT proteins (Fig. 3.5).

3.6 *Lhk* transcripts possess overlapping expression profiles

To gain insight into expression profiles of the *Lhk* genes in *L. japonicus,* semi-quantitative RT-PCR was employed to assess steady-state levels of all *Lhk* mRNAs in various *L. japonicus* tissues. *Lhk* transcripts were detected in all major organs analyzed, with some apparent differences between tissues and between *Lhk* transcripts (Fig. 3.6). The closely-related *Lhk1* and *Lhk1A* displayed similar expression profiles, being most abundant in roots and nodules and not as abundant in leafs. *Lhk2* was highly expressed in roots, but less so in nodules. In contrast, *Lhk3* was expressed at a low level in roots and more so in nodules and leaves. These results indicate that although the *Lhk1, 1A, 2 and 3*

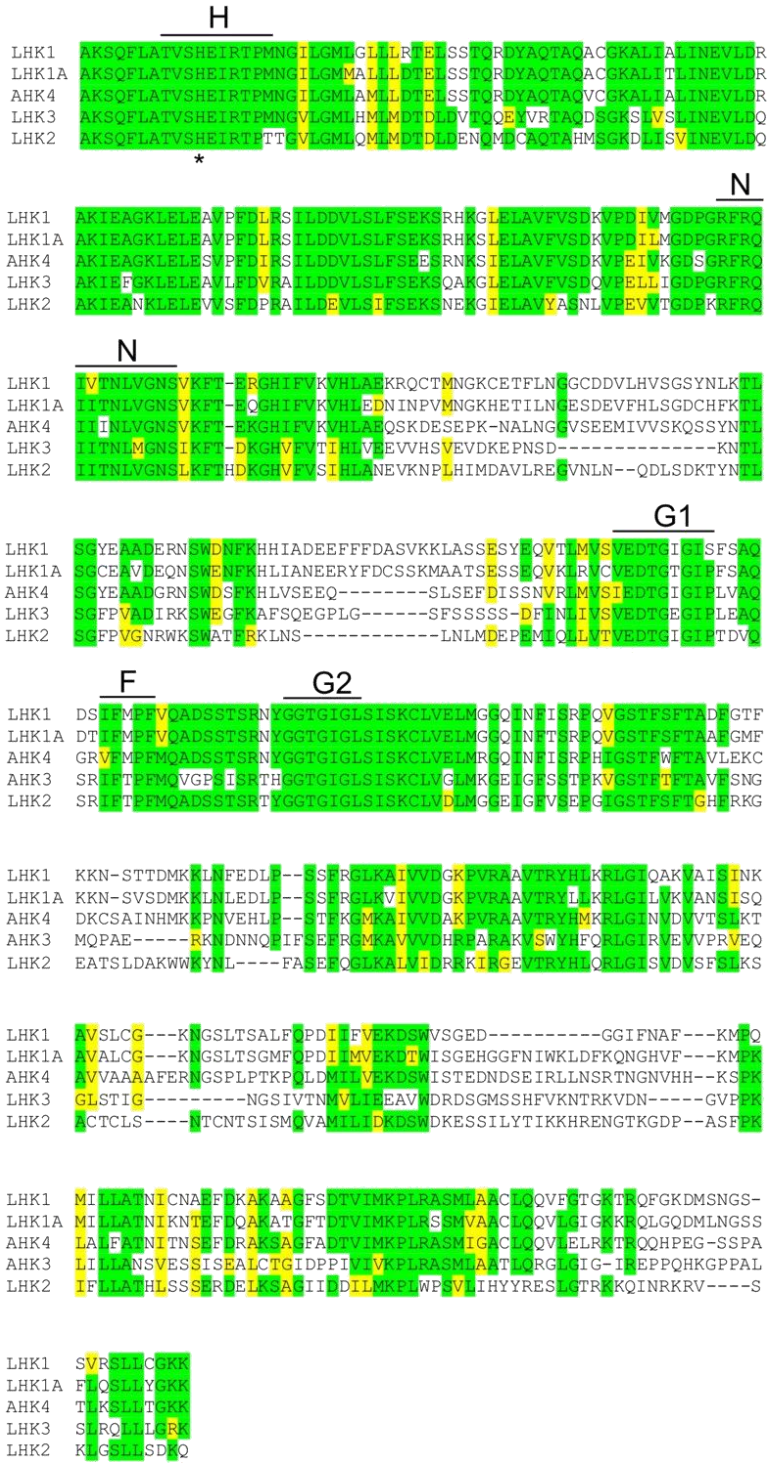


Figure 3.4 Alignment of the LHK kinase domain. The conserved H, N, G1, F, and G2 box motifs are indicated. An asterisk denotes the presence of a conserved histidine (H) residue required for the phosphorelay. The alignment was created as before (see Fig. 3.3).

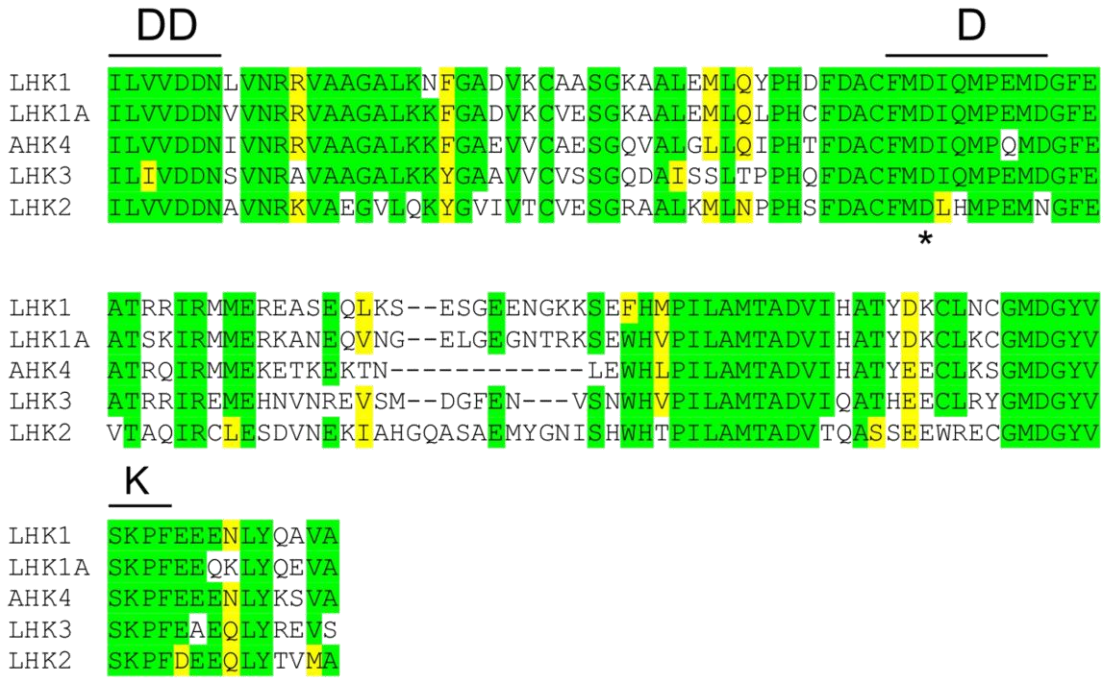


Figure 3.5 Alignment of LHK receiver domains. The conserved DD, D, and K motifs are indicated. An asterisk denotes the presence of conserved aspartic acid (D) residue required for the phosphorelay. The alignment was created as before (see Fig. 3.3).

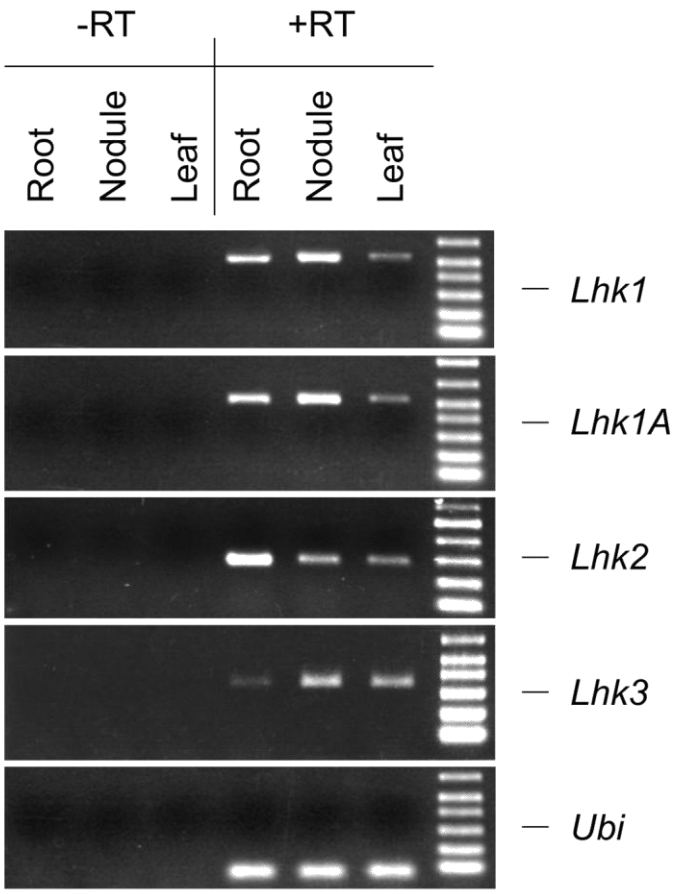


Figure 3.6 Semi-quantitative RT-PCR of *Lhk* transcripts in *L. japonicus*. The steady-state level of *Lhk* transcripts in various *L. japonicus* tissues is shown along with the outcome of PCR amplification reactions without prior reverse transcription step (-RT) and the ubiquitin mRNA controls. In all cases, a gene-specific RT-PCR product which encompassed a portion of the final exon and the 3' UTR was amplified and sequenced to ensure transcript specificity.

transcripts are ubiquitously present in all *L. japonicus* organs tested, some tissue-enhanced expression could be detected (Fig. 3.6), similar to the expression patterns described for cytokinin receptor gene family in *A. thaliana* (Higuchi *et al.*, 2004; see also Discussion).

3.7 The *Lhk3* transcript undergoes alternative splicing

While performing pair-wise comparisons between LHK proteins and their presumed counterparts in *A. thaliana*, a significant difference at the N-terminus was detected between the predicted LHK3 and the *A. thaliana* AHK3 protein; this prompted additional investigation. By using 5'-RACE approach and total RNA derived from *L. japonicus* nodules, two variants (variant 1 and 2) of the *Lhk3* mRNA were identified (Fig. 3.7). These variants differed in length, with variant no. 2 being extended by 228 bp at its 5'end in comparison with *Lhk3* mRNA variant no. 1. In order to solidify the 5'-RACE result, transcript-specific primers were designed against the each predicted *Lhk3* mRNA variant and the presence of both mRNA species was confirmed via RT-PCR in *L. japonicus* nodules (Fig. 3.7B). After sequencing each product, the mRNA variant no. 2 was found to be longer, resulting in the addition of 51 amino acids at the N-terminal end of its predicted translational product. When aligned to AHK3, the translational product of *Lhk3* mRNA for variant no. 2 produced an improved alignment over variant no. 1 (Fig. 3.7C).

As the initial RT-PCR experiment indicated differences in the relative abundance of the two *Lhk3* mRNA variants in nodules, a semi-quantitative RT-PCR was again employed to analyze whether or not their abundance could be regulated in any way by infection

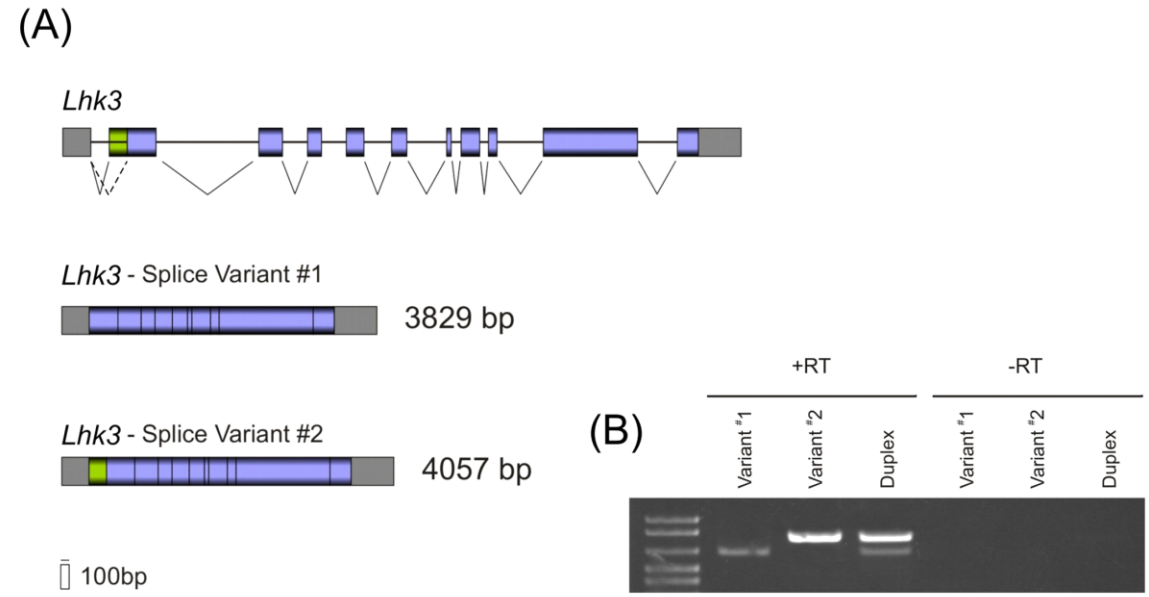


Figure 3.7 *Lhk3* is alternatively spliced. (A) Alternative splicing at the intron1/exon 2 junction of the *Lhk3* locus results in the production of two *Lhk3* splice variants (named *Lhk3* - Splice Variant #1 (dashed line) and #2 (solid line), differing by 228bp (green box). (B) RT-PCR and transcript-specific primers were used to detect and sequence these two splice variants in *L. japonicus* nodules. (C) Alignment of the N-terminal portion for the two predicted LHK3 protein variants with the corresponding protein region of *A. thaliana* AHK3. The alignment was created as before (see Fig. 3.3) with a threshold set at 65%.

with *M. loti*, or by subsequent nodule development. To simultaneously assess the expression of both *Lhk3* splice variants, a duplex PCR was conducted (see Section 2.5). Both transcripts were detected at all stages during nodule development and in mature nodules (Fig. 3.8). Consistent with the previous experiment (Fig. 3.7B), the relative abundance of mRNA variant no. 2 was higher in comparison to mRNA variant no. 1 and this did not change at any time-point/tissue analyzed. This result suggested therefore, that symbiotic interaction is unlikely to influence the relative abundance of *Lhk3* splicing variants.

3.7 *Lhk1A* and *Lhk3* code for functional cytokinin receptors

3.7.1 *Lhk1A* confers cytokinin-responsive growth to the *sln1Δ* mutant of *S. cerevisiae*

To evaulate the cytokinin-specific function of LHKs identified in *L. japonicus*, the well-characterized *sln1Δ* HK mutant (a galactose auxotroph) of *S. cerevisiae* was used (Maeda *et al.,* 1994). This mutant yeast strain was previously employed to demonstrate cytokinin responsive function of the LHK1 receptor (Murray *et al.,* 2007). Following transformation, the wild-type *Lhk1A* cDNA was able to restore growth of the *sln1Δ* strain in a cytokinin-dependent fashion (Fig. 3.9). Repeated attempts to perform similar assays with *Lhk2* and the two variants of the *Lhk3* cDNA have failed due to apparent toxicity of these cDNAs in the yeast cells (not shown). Therefore, an alternative approach based on a two-component phosphorelay assay developed in *E. coli* (Yamada *et al.,* 2001) was used.

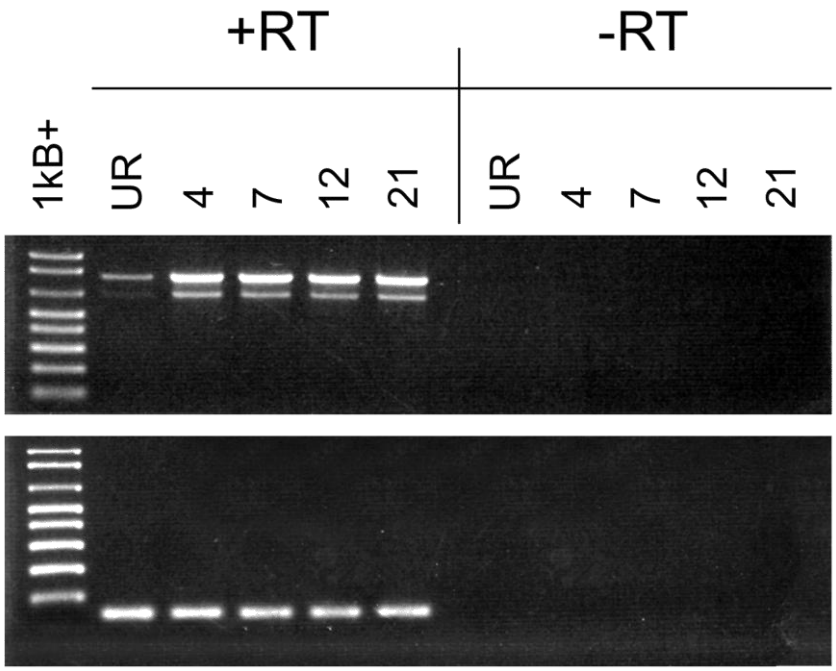


Figure 3.8 Steady-state level of *Lhk3* transcripts during nodule development. The top panel depicts the result of duplex, semi-quantitative RT-PCR for both splice variants in *L. japonicus* uninoculated wild-type roots (UR) and nodules collected 4, 7, 12, and 21 DAI with *M. loti*. The cDNA fragment for the *Lhk3* mRNA variant no. 2 is larger in size (786 bp), while the corresponding cDNA for the splicing variant no. 1 is represented by the lower molecular mass band (641 bp). Although the total difference between *Lhk3* splice variants is 228 bp, the image depicts 145 bp difference only due to transcript-specific primer design. The bottom panel represents amplification products for the ubiquitin mRNA, used as a positive control.

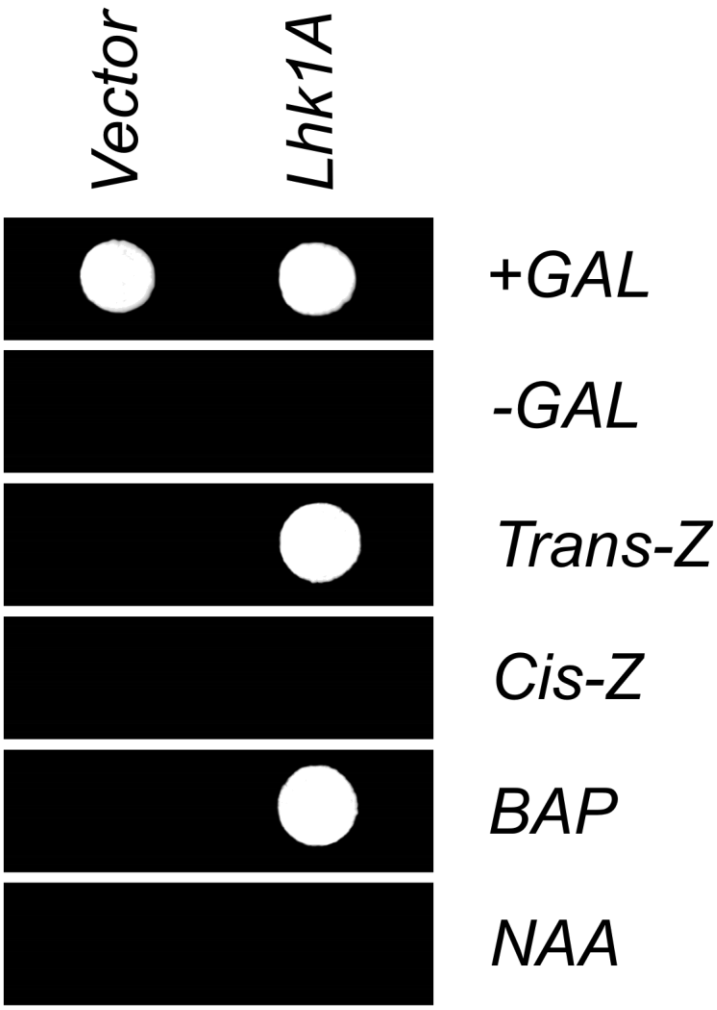


Figure 3.9 Functional analysis of *Lhk1A*. The wild-type *Lhk1A* cDNA confers cytokinin (Trans-Z and BA)-dependant rescue of yeast growth in the absence of galactose (-GAL) when transformed into the *sln1Δ* mutant of *S. cerevisiae*. Non-specific ligands do not restore yeast growth (Cis-Z and NAA). + Gal, 2% galactose supplement; Trans-Z, trans-zeatin; Cis-Z, cis-zeatin; BAP, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid.

3.7.2 *Lhk3* confers cytokinin-responsive growth to the sensor-negative SRC122 mutant of *E. coli*

In addition to *Lhk1* and *Lhk1A*, which were shown to confer cytokinin-dependent rescue of the *sln1Δ* yeast mutant growth and, therefore, were defined as functional cytokinin receptors (Murray *et al.*, 2007 and this work), two additional loci, *Lhk2* and *Lkh3* remained to be functionally evaluated. Given the failure of the yeast system, the sensor-negative SRC122 (*ΔrcsC*) mutant of *E. coli* was utilized for this purpose. The presence of a functional HK receptor and the appropriate ligand in the SRC122 *E. coli* results in the activation of a *cps::lacZ* fusion. This provides both visible and also quantifiable assay based on the *β*-galactosidase (LacZ) reporter activity, which is inferred via the cleavage of the colorimetric ortho-Nitrophenyl-β-galactoside (ONPG) substrate to O-nitrophenol (Yamada *et al.*, 2001). Application of cytokinin to the SRC122 *E. coli* strain transformed with wild-type copies of either of the two splice variants of *Lhk3* significantly induced the *β*-galactosidase activity above the control level in the untreated samples, thus confirming the cytokinin-responsive function of these receptors (Fig. 3.10). In contrast to *Lhk3*, the *Lhk2*-containing replicon showed signs of instability when transformed into SRC122. This was reflected by DNA rearrangements (Fig. 3.11) that caused damage to the *Lhk2* open-reading frame (not shown). This outcome was not surprising given the initial observations made in yeast cells and also the presence of related reports on similar problems in *A. thaliana* (Yamada *et al.*, 2001). Given these results, the LHK2 receptor remains undefined and continues to be considered here after as a presumed cytokinin receptor. In the same context, none of the mutations in the *Lhk2* locus identified by the TILLING approach could be functionally evaluated.

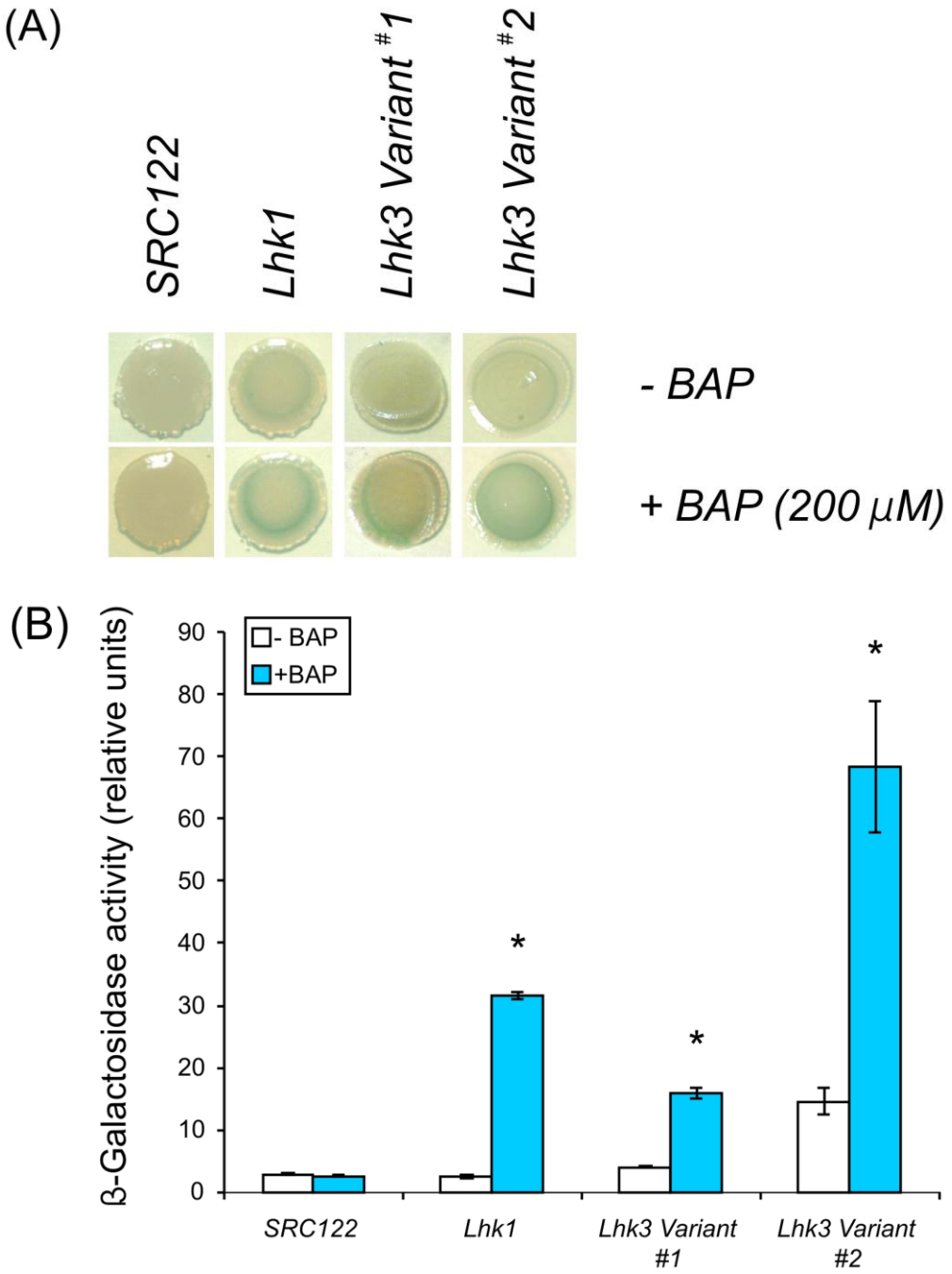


Figure 3.10 *Lhk3* codes for a functional cytokinin receptor. The full-length cDNAs for the two alternative splice variants produced by *Lhk3* were cloned into the pSTV28 expression vector and transformed into sensor-negative SRC122 *E. coli*. (A) A noticeable increase in the formation of blue color can be observed when bacteria were plated in the presence of X-gal and 200 μM BAP. (B) Relative units of *β*-galactosidase activity from different *Lhk* constructs in the presence or absence of 200 μM BAP. In all cases, values represent the mean ± 95% CI (n = 3). An asterisk denotes significant differences (Student's t-Test, $P<0.05$). The *Lhk1* cDNA was used as a positive control for these experiments.

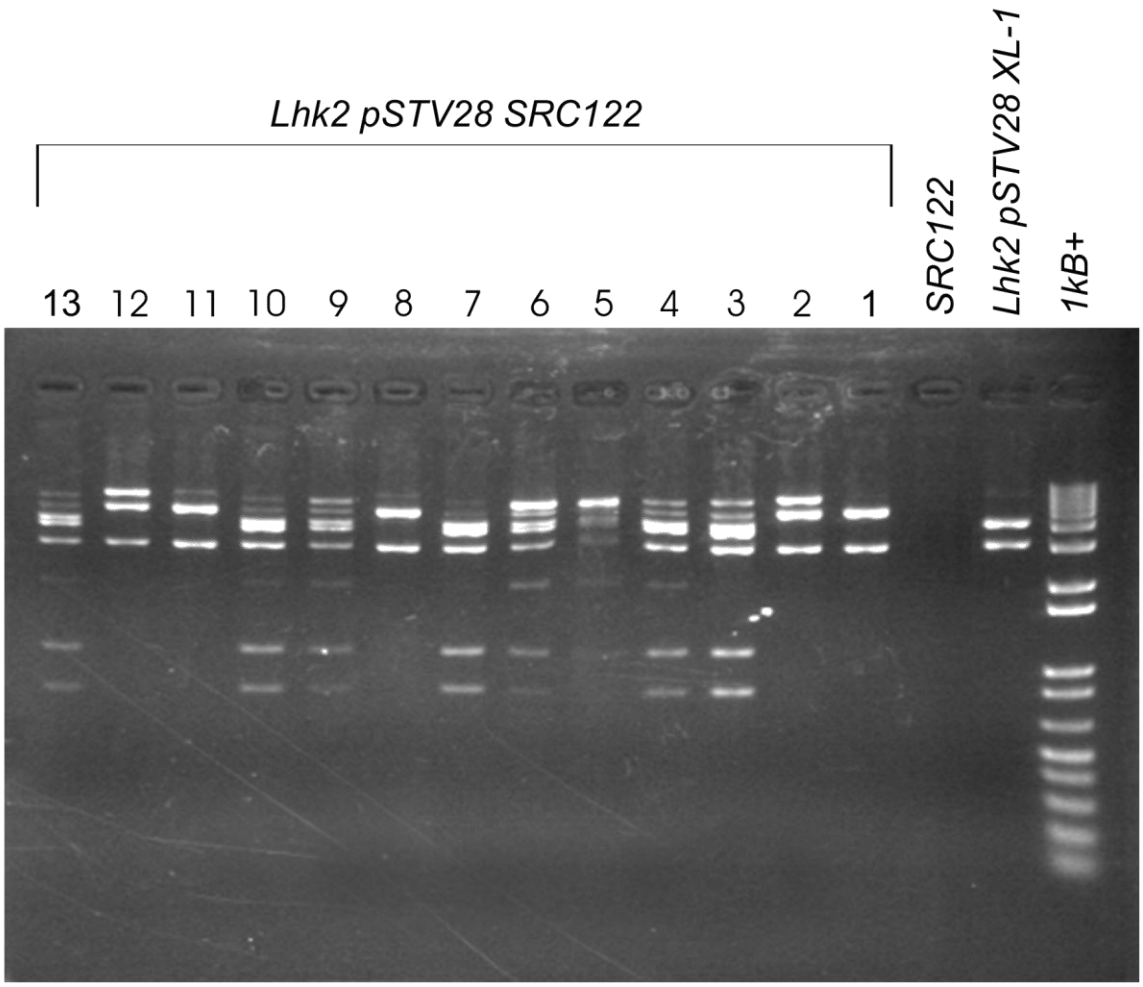


Figure 3.11 Rearrangements of the *pSTV28-Lhk2* plasmid in the SRC122 strain. The full-length *Lhk2* cDNA was cloned directionally into the *pSTV28* expression vector and subsequently transformed into the *XL-1* Blue *E. coli* strain. Double digestion of this vector with *Eco*RI and *Sal*I was predicted to release the *Lhk2* cDNA (~3.8 kB) from the pSTV28 vector (~3 kB), as shown in the *Lhk2 pSTV28 XL-1* control lane. When transformed into the SRC122 *E. coli* mutant strain, the *Lhk2 pSTV28* plasmid showed rearrangements (*Lhk2 pSTV28 SRC122* colonies #1-13), as indicated by altered restriction patterns. As a further control, plasmid DNA was isolated from the nascent SRC122 strain without transformation to ensure that no additional plasmids were contributing to the additional bands observed for *Lhk2 pSTV28 SRC122* colonies. The control SRC122 lane clearly shows that these *E. coli* cells do not carry any additional plasmids.

3.8 Isolation of *lhk* TILLING mutants

To address the functional significance of the *Lhk* gene family during symbiosis, a reverse genetics approach, Targeted Induced Localized Lesions IN Genomes (TILLING), was used to identify mutations in the *Lhk1A, Lhk2 and Lhk3* loci. The identification of such mutations would complement the already described, loss-of-function mutations in *Lhk1* (see Murray *et al.,* 2006), thus allowing for a more comprehensive, functional characterization of this small gene family with regard to its possible contribution to symbiosis.

A search for mutations using the TILLING approach was set to target a 1 kB region within the highly conserved and functionally required kinase domain. This was done to increase the likelihood of identifying mutations deleterious to the function of the resulting protein. Several ethylmethyl sulfonate (EMS)-mutagenized *L. japonicus* lines that carried single nucleotide substitutions within the *Lhk1A*, *Lhk2*, and *Lhk3* loci were successfully identified (Fig. 3.12). For *Lhk1A*, a mutant line that carried a G_{1695} to A transition was chosen for further detailed analyses (the mutant allele named *lhk1a-1*). This nonsense mutation was predicted to change a tryptophan residue in the kinase domain to a premature stop codon (W_{564} to STOP). For the *Lhk2* and *Lhk3* loci, a number of single nucleotide transitions that resulted in amino acid substitutions were discovered. Among these, the *lhk2-5* (G_{834} to R) and *lhk3-1* (R_{561} to Q) mutant lines were selected for subsequent analyses due to their affliction of an invariant residue within the conserved G1 and N box motifs of the kinase domain, respectively (Fig. 3.13).

Mutant Line	Mutation (gDNA)	ORF change
lhk1a-1	G_{4439} - A	W_{565} - STOP
lhk1a-2	C_{4808} - T	L_{689} - F
lhk1a-3	C_{4808} - T	L_{689} - F
lhk1a-4	G_{4725} - A	G_{661} - E
lhk1a-5	G_{4929} - A	S_{729} - N
lhk1a-6	C_{4388} - T	H_{549} - T
lhk1a-7	G_{3934} - A	None - Intronic
lhk1a-8	C_{4504} - T	None - synonomous
lhk1a-9	C_{4031} - T	None - Intronic
lhk1a-10	G_{4933} - A	None - synonomous

Mutant Line	Mutation (gDNA)	ORF change
lhk2-1	G_{5499} - A	A_{517} - T
lhk2-2	G_{5532} - A	D_{528} - N
lhk2-3	G_{5547} - A	E_{533} - K
lhk2-4	G_{5551} - A	G_{534} - D
lhk2-5	G_{5769} - A	G_{605} - R
lhk2-6	G_{5817} - A	A_{621} - T
lhk2-7	G_{5836} - A	R_{627} - Q
lhk2-8	G_{5899} - A	G_{648} - E
lhk2-9	G_{5908} - A	G_{651} - E
lhk2-10	G_{6051} - A	D_{699} - N
lhk2-11	C_{5645} - T	None - synonomous
lhk2-12	C_{5248} - T	None - Intronic
lhk2-13	C_{5335} - T	None - synonomous
lhk2-14	C_{5691} - T	None - synonomous
lhk2-15	G_{5260} - A	None - Intronic

Mutant Line	Mutation (gDNA)	ORF Change
lhk3-1	G_{4558} - A	R_{561} - Q
lhk3-2	G_{5219} - A	V_{596} - I
lhk3-3	G_{6057} - A	S_{875} - N
lhk3-4	C_{4782} - T	None - Intronic
lhk3-5	G_{4973} - A	None - Intronic

Figure 3.12 *Lhk* mutant alleles as identified by the TILLING approach. Genetic lesions induced by EMS mutagenesis and identified by TILLING within the conserved kinase domains are shown (see Section 2.6). gDNA: a specific nucleotide substitution detected in the genomic DNA for a given locus; ORF change: the resulting alteration to the open reading frame of a given protein. Adenine in the predicted ATG initiation codon was set as 1 for defining the position of a given mutation in gDNA. Similarly, a predicted initiating methionine residue was set as 1 to calculate the position of any given amino-acid change.

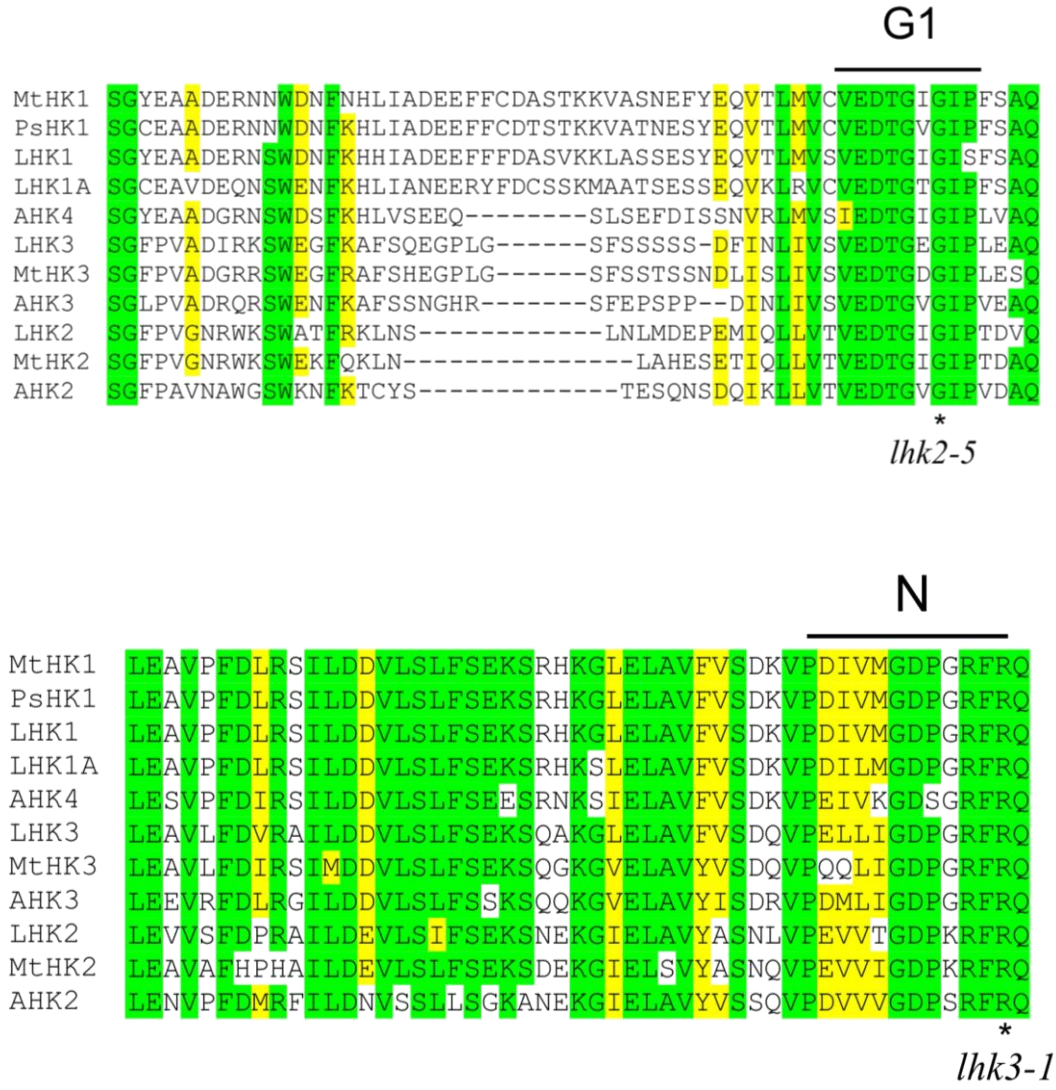


Figure 3.13 Selected TILLING mutants obtained for *Lhk2* and *Lhk3*. *lhk2-5* (G_{834} to R) and *lhk3-1* (R_{561} - Q) carry genetic lesions in absolutely conserved residues (asterisks) within the predicted G1 and N box motifs of the kinase domain, respectively. The alignment was created as before (see Fig. 3.3). LHK: *L. japonicus* MtHK: *M. truncatula*; PsHK: *P. sativum*; AHK: *A. thaliana* histidine kinase.

3.9 The *lhk1a-1* and *lhk3-1* mutations are deleterious to cytokinin responsive function of *Lhk1A* and *Lhk3*

The cDNA corresponding to the *lhk1a-1* mutant allele was functionally evaluated in the same manner as the wild-type *Lhk1* cDNA by utilizing the *sln1Δ* HK mutant of *S. cerevisiae* (Fig. 3.14). The *lhk1a-1* cDNA failed to restore cytokinin-dependent growth of *sln1Δ* thus, unequivocally demonstrating the deleterious nature of the *lhk1a-1* mutation.

In an independent experiment, site-directed mutagenesis was performed to reconstruct the *lhk3-1* mutation in wild-type variants of *Lhk3*. When assayed in the bacterial system outlined in section 3.7.2, the *lhk3-1* mutation completely abolished the cytokinin responsiveness of the LHK3 receptor, regardless of the cDNA variant used, indicating that this mutation is indeed deleterious to the receptor function (Fig. 3.15).

3.10 Symbiotic phenotypes of *lhk* TILLING mutants

3.10.1 Symbiotic interaction with *M. loti* is largely unaffected in *lhk1a-1*, *lhk2-5*, and *lhk3-1* single mutant backgrounds

To assess the possible roles of *Lhk1A*, *Lhk2*, and *Lhk3* during the NFS, phenotypic analysis of the selected TILLING mutant lines carrying deleterious mutations in the aforementioned loci were conducted. Early events that are characteristic of the epidermal program, such as the formation of bacterial microcolonies trapped within curled root hairs and subsequent production of ITs, were evaluated seven DAI with *M. loti* strain carrying the *hemA*: *lacZ* reporter gene fusion. The *lhk1a-1*, *lhk2-5* and *lhk3-1* mutants showed no significant deviation from the wild-type phenotype with regards to these features (Fig. 3.16). Nodule organogenesis in the *lhk1a-1*, *lhk2-5* and *lhk3-1* single

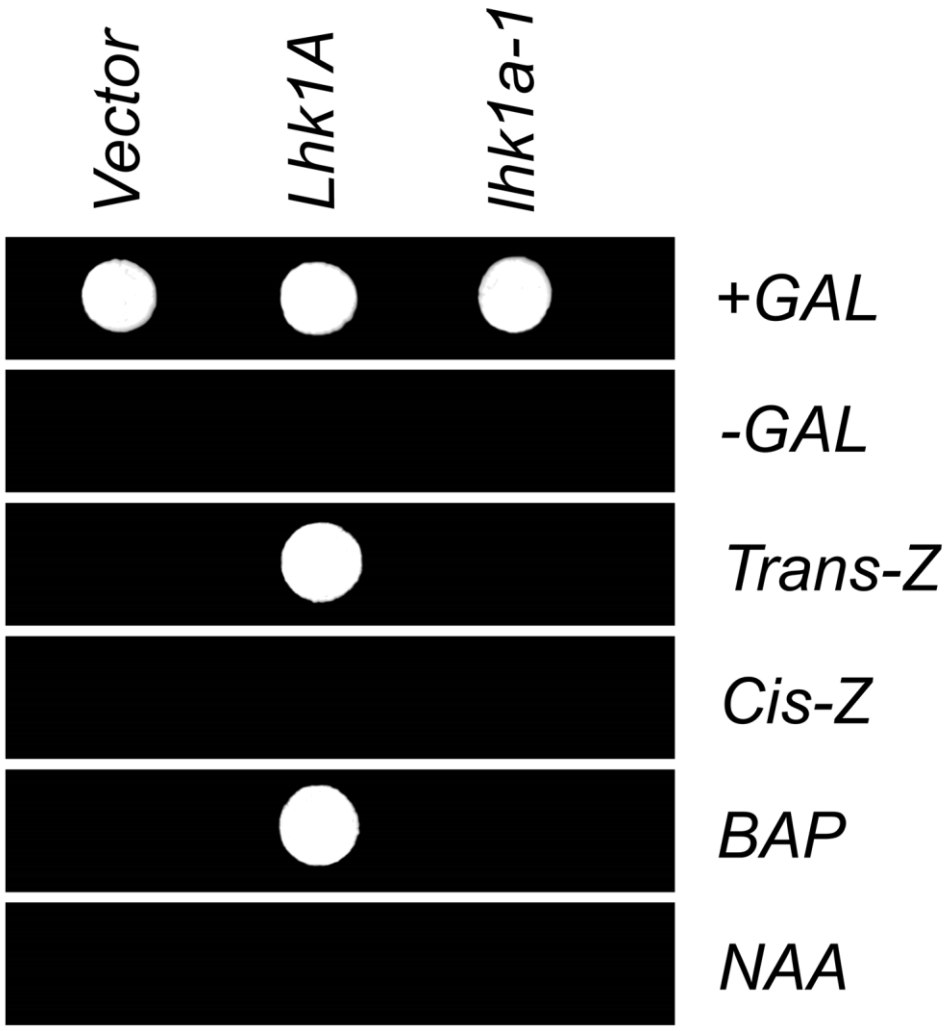


Figure 3.14 The *lhk1a-1* TILLING mutant is loss-of-function. Contrasting the wild-type scenario, the *lhk1a-1* mutant cDNA failed to rescue the cytokinin-dependant growth when transformed into the *sln1Δ* mutant of *S. cerevisiae* in the absence of galactose (GAL) and in the presence of the appropriate ligand (BA and Trans-Z). Non-specific ligands also did not restore yeast growth (Cis-Z and NAA). + Gal, 2% galactose supplement; Trans-Z, trans-zeatin; Cis-Z, cis-zeatin; BA, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid.

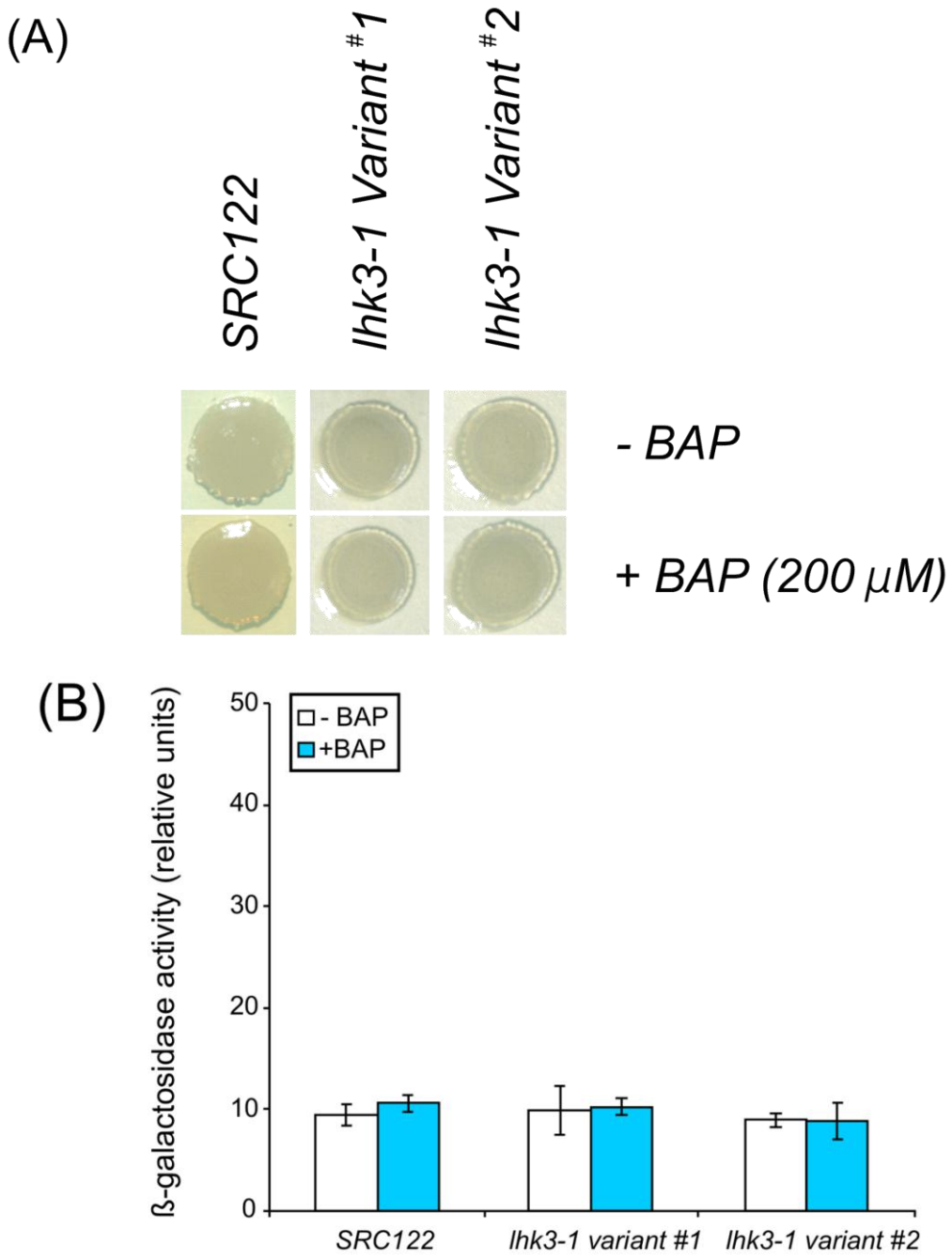
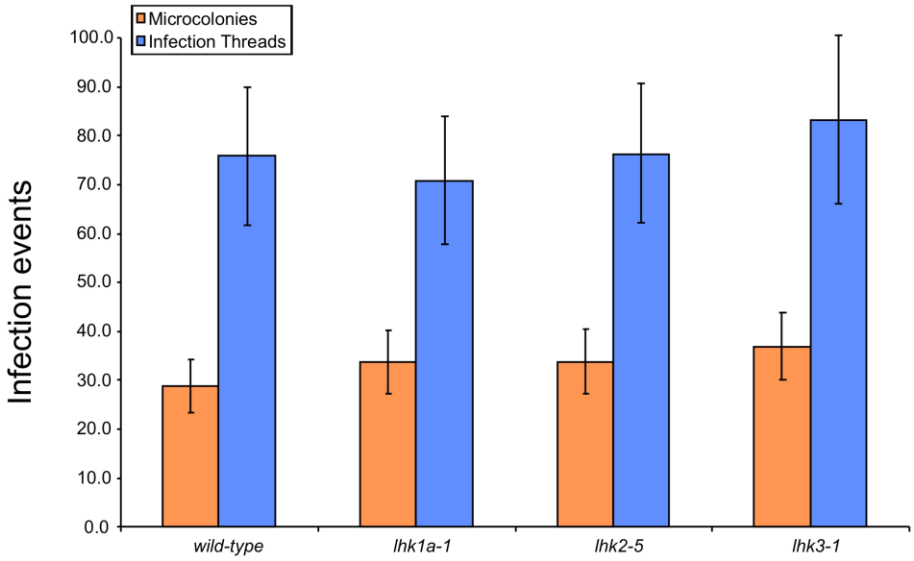


Figure 3.15 The *lhk3-1* mutation abolishes the cytokinin-responsive function of the LHK3 receptor. Site-directed mutagenesis was used to recreate the *lhk3-1* mutation in both of the two splice variants produced by *Lhk3*. These were subsequently cloned into the pSTV28 expression vector and tested in the sensor-negative SRC122 *E. coli*. (A) In contrast to the wild-type *Lhk3* cDNA (see Fig.3.9), the mutant *Lhk3* variants failed to induced the β-galactosidase reporter activity above background levels in the presence of X-gal and 200 μM BAP l (B) Relative units of *β*-galactosidase activity from different *Lhk* constructs in the presence or absence of 200 μM BAP. In all cases, values represent the mean ± 95% CI (*n* = 3).



Line	Microcolonies	Infection Threads
wild-type	28.8 ± 5.41	75.9 ± 14.18
lhk1a-1	33.7 ± 6.7	70.9 ± 13.0
lhk2-5	33.8 ± 6.6	76.4 ± 14.3
lhk3-1	37.0 ± 7.0	83.3 ± 17.2

(n = 10)

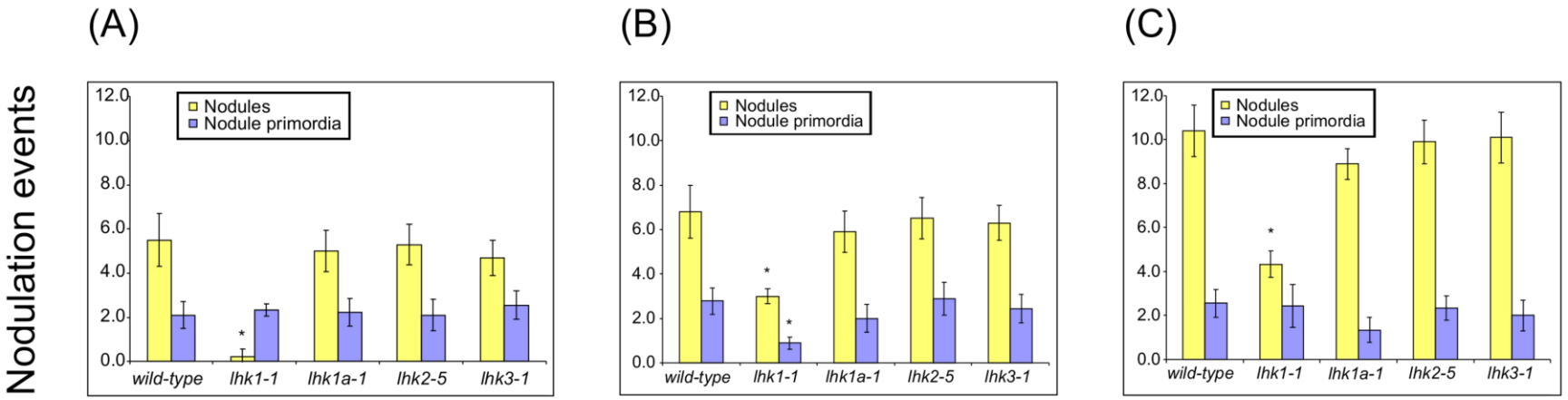
Figure 3.16 Bacterial infection is unaffected in *lhk1a-1*, *lhk2-5* and *lhk3-1* mutants. The formation of microcolonies and ITs were scored in *lhk* single mutants at 7 DAI and compared to the wild-type control. In all cases, values reported are the mean ± 95% CI (*n = 10*).

mutant lines was also unaltered when evaluated at various time-points after inoculation (Fig. 3.17A, B, C). For example, eight to ten pink nodules were formed on average by *lhk1a-1*, *lhk2-5* and *lhk3-1* mutant roots at 21 DAI. This was not significantly different from the number of functional nodules formed by the wild-type control plants. As expected, the *lhk1-1* mutant formed a significantly reduced number of nodule structures when analysed at the same time point (Fig. 3.17A, B).

Histochemical staining for the β-galactosidase reporter activity showed that like in wild-type plants, nodules that formed on the roots of *lhk1A-1*, *lhk2-5* and *lhk3-1* mutants were colonized by *M. loti*, as indicated by the dark blue color within nodule structures. No excessive infection of the root epidermis by *M. loti* in these mutant lines was observed, which was in contrast with the hyperinfection phenotype of the *lhk1-1* roots (Fig. 3.18). The *lhk1-1* phenotype was consistent amongst mutant alleles tested in addition to *lhk1a-1*, *lhk2-5*, and *lhk3-1* including *lhk1a-2*, *lhk1a-5*, *lhk2-7*, *lhk2-8*, *lhk2-10*, *lhk3-2*, and *lhk3-3*. Nodulation events (nodules and nodule primordia) were scored at 21 DAI and found to be not significantly different from the wild-type (not shown).

3.10.2 *lhk* double mutants carrying the loss-of-function *lhk1-1* allele phenocopy *lhk1-1* single mutants

To further assess whether or not the *Lhk1A*, *Lhk2*, and *Lhk3* loci participated in the NFS, different *lhk* single mutants were crossed into the *lhk1-1* mutant background. All crosses were successfully achieved; however, growth of the F1 plant derived from the cross between *Lhk2* and *Lhk1-1* was very slow and, thus far, the production of F2 seeds was not sufficient to perform further analyses.



7 DAI

Line	Nodules	Nodule primordia
wild-type	5.5 ± 0.9	2.1 ± 0.7
lhk1-1	0.2 ± 0.3	2.3 ± 0.4
lhk1a-1	5.0 ± 0.7	2.2 ± 0.4
lhk2-5	5.3 ± 1.0	2.1 ± 0.7
lhk3-1	4.7 ± 6	2.6 ± 0.8

(n = 10)

14 DAI

Line	Nodules	Nodule primordia
wild-type	6.8 ± 1.2	2.8 ± 0.6
lhk1-1	3.0 ± 0.3	0.89 ± 0.3
lhk1a-1	5.9 ± 0.9	2.0 ± 0.6
lhk2-5	6.5 ± 0.9	2.9 ± 0.7
lhk3-1	6.3 ± 0.8	2.4 ± 0.6

(n = 10)

21DAI

Line	Nodules	Nodule primordia
wild-type	10.4 ± 1.2	2.6 ± 0.6
lhk1-1	4.3 ± 0.6	2.4 ± 1.0
lhk1a-1	8.9 ± 0.7	1.3 ± 0.6
lhk2-5	9.9 ± 1.0	2.3 ± 0.6
lhk3-1	10.1 ± 1.2	2.0 ± 0.6

(n = 10)

Figure 3.17 Nodule organogenesis is unaffected in *lhk1a-1*, *lhk2-5* and *lhk3-1* single mutant backgrounds. Nodule and nodule primordia formation in *lhk1-1*, *lhk1a-1*, *lhk2-5*, and *lhk3-1* single mutants was scored at (A) 7 DAI, (B) 14 DAI, and (C) 21 DAI. The *lhk1-1* mutant was used for comparison. In all cases, values reported are the mean ± 95% CI (*n = 10*). An asterisk denotes significant differences (Student's t-Test, $P<0.05$).

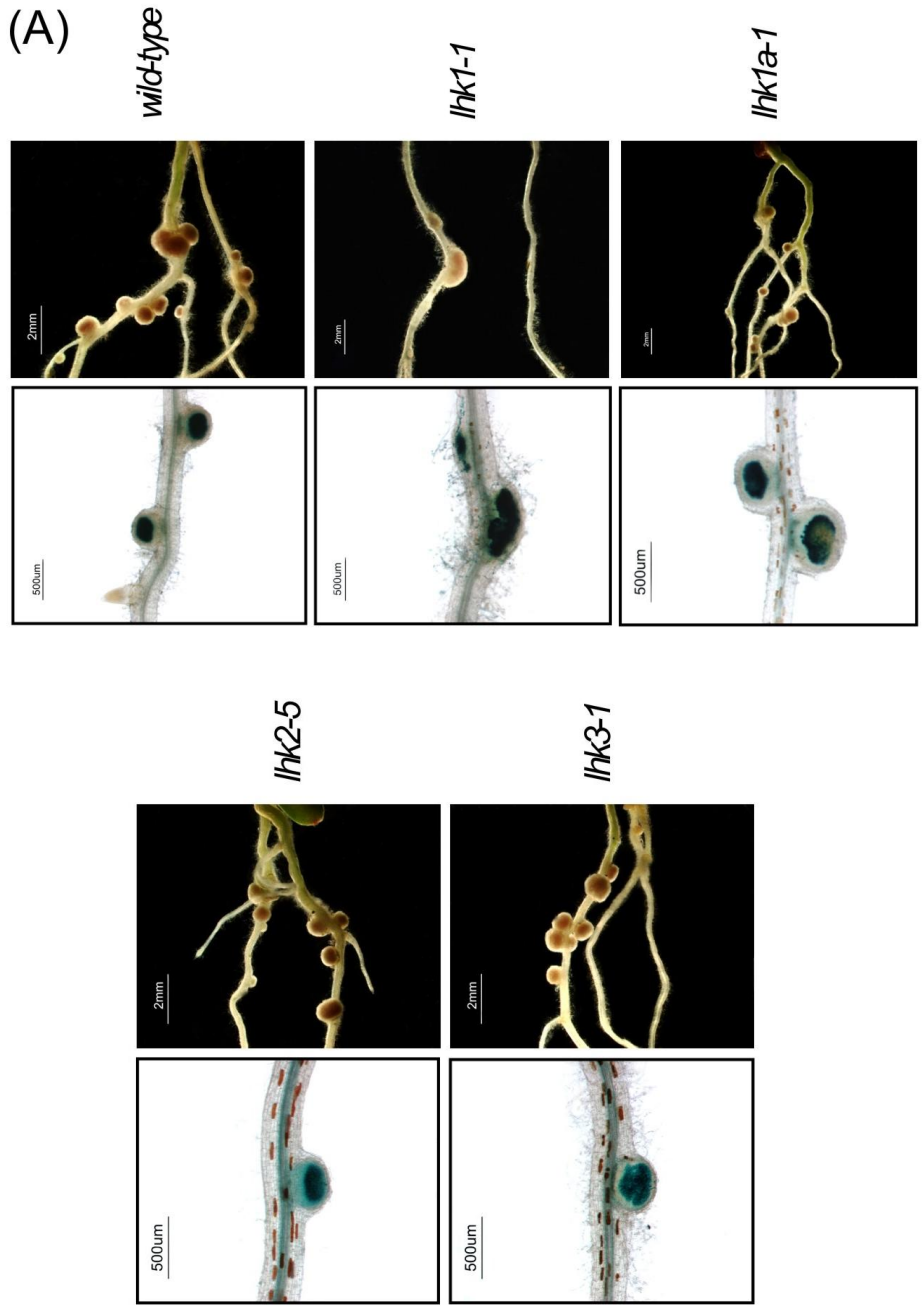


Figure 3.18 Symbiotic phenotype of *lhk1a-1*, *lhk2-5*, and *lhk3-1* mutants. Representative images of the nodulated root at 21 DAI for each line prior to staining (black background) and following histochemical staining for *β*-galactosidase activity (white background). The *lhk1-1* mutant was used for comparison. Blue color reflects the *β*-galactosidase activity, which also indicates the presence of bacteria.

As shown Fig. 3.19, *lhk1a-1 lhk1-1* or *lhk3-1 lhk1-1* double mutants produced a symbiotic phenotype which mimicked the *lhk1-1* single mutant. At the whole root level, these double mutants formed only few, oblong nodules in a manner reminiscent of the *lhk1-1* single mutant phenotype (Fig. 3.19A, B); they were also hyperinfected at the root epidermis, as observed in *lhk1-1* (not shown). Furthermore, when the *lhk1a-1* mutant was crossed to *lhk3-1*, the resulting double mutant displayed a wild-type nodulation pattern, forming a wild-type number of ITs (not shown) and fully infected nodules (Fig. 3.19B).

3.10.3 Symbiotic interaction with AM fungi is unaltered in *lhk* mutants

In addition to symbiotic interactions with N-fixing bacteria, phosphate acquisition is supplemented in *L. japonicus* via a mutualistic interaction with AM fungi of the phylum *Glomeromycota* (Bonfante and Genre, 2010). In contrast to NFS, where root nodules are formed, the interaction with AM fungi does not involve the formation of a new plant organ. In order to address the question whether or not the LHK cytokinin receptors are required for AM symbiosis, *L. japonicus* wild-type and *lhk* mutant lines were inoculated with a mixture of *Glomus sp.* and scored for fungal infection and the presence of structural features, such as extra- and intra-radical hyphae, vesicles and arbuscules, that are characteristic of the wild-type interaction. As shown in Figure 3.20, all *lhk* mutant lines tested were unaffected in their ability to interact with the fungi.

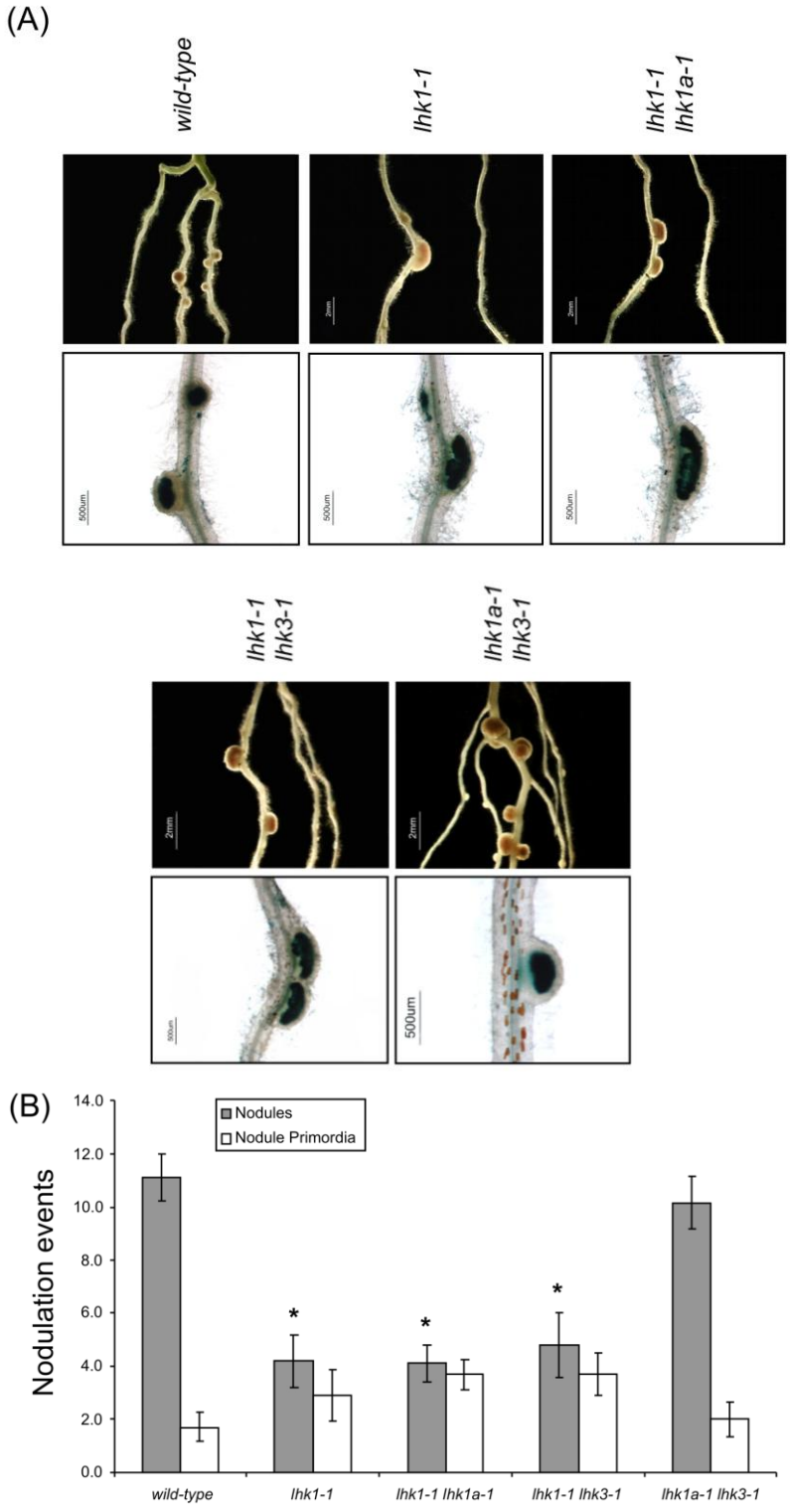


Figure 3.19 Symbiotic phenotype of *lhk* double mutant lines. (A) Nodule organogenesis on *L. japonicus* roots prior to (black background) and following histochemical staining for the *hemA*:*lacZ*-tagged rhizobia (white background) is shown at 21 DAI. (B) Nodulation events scored at 21 DAI from the corresponding lines. In all cases, values represent the mean ± 95% CI (*n = 10*).

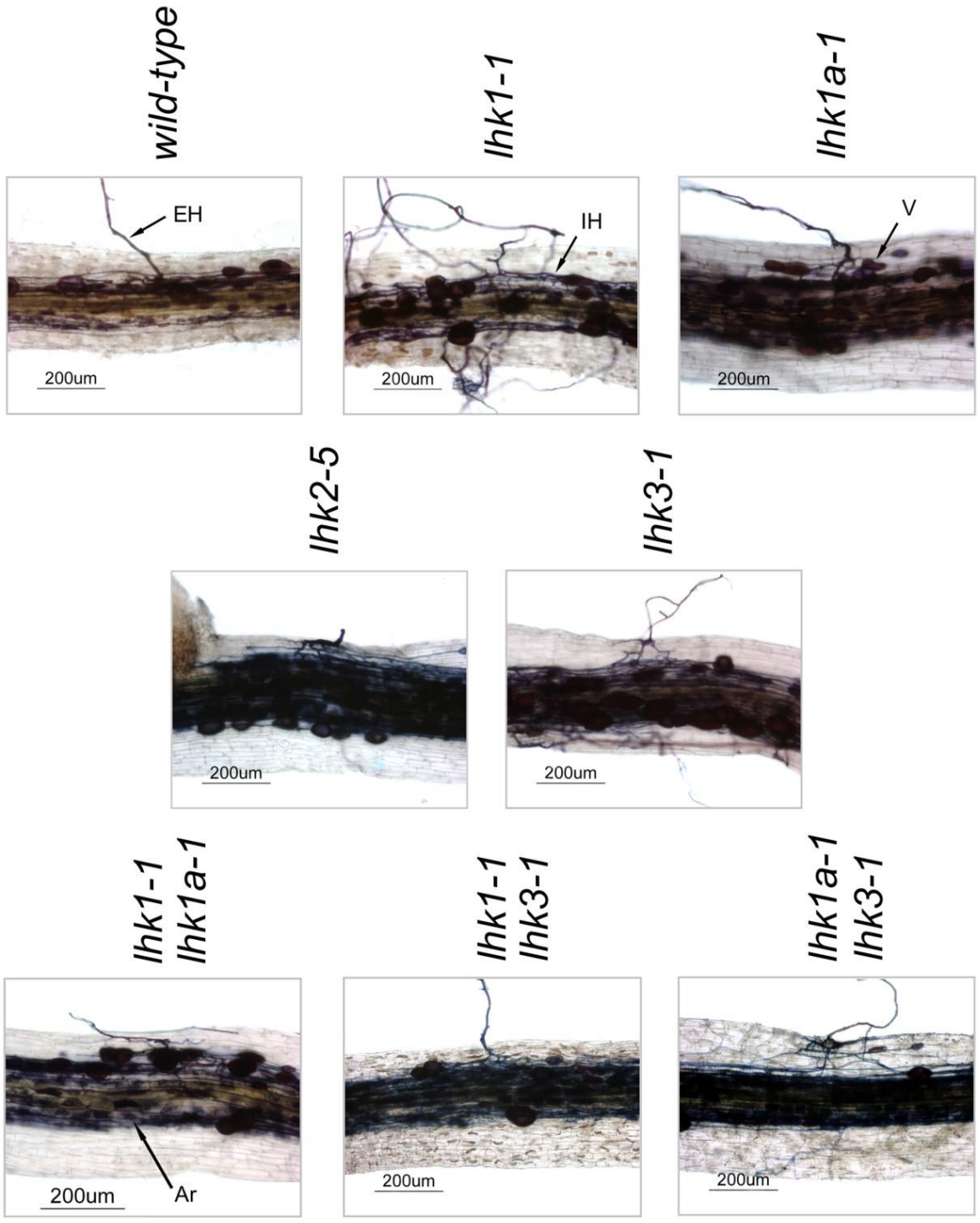


Figure 3.20 Symbiotic interaction of *L. japonicus* roots with AM fungi is unaffected in *lhk* mutant backgrounds. Representative images of colonized roots eight weeks post-inoculation are shown for the collection of *lhk* single and double mutants and the wild-type *L. japonicus*. EH, Extracellular hyphae; IH, Intracellular hyphae; V, vesicle; Ar, Arbuscle.

3.11 Response of *lhk* mutants to exogenous cytokinin application

In wild-type *L. japonicus* plants, root elongation is significantly inhibited by external application of cytokinin, such as BAP. This effect is clearly observable even at the concentration of BAP as low as 1x 10^{-8} M. At higher BAP concentrations, wild-type roots elongate only to roughly 20% of the untreated control (Fig. 3.21A).

Deleterious mutations in the *Lhk1* receptor, such as *lhk1-1*, were shown to render mutant roots strongly insensitive to exogenously applied cytokinin (Murray *et al.*, 2007). These data indicated, therefore, that in addition to its significant role in nodule organogenesis, the LHK1 receptor mediates root responses to external (environmental) signals, such as cytokinin.

To analyze whether or not other *L. japonicus* cytokinin receptors partake in this physiological response, all *L. japonicus lhk* mutant lines were subjected to the same root elongation assay. The *lhk1-1* mutant and wild-type *L. japonicus* were used as controls. In contrast to *lhk1-1*, which exhibited strong cytokinin insensitivity, the *lhk1a-1*, *lhk2-5*, and *lhk3-1* mutants responded to exogenous treatment with BAP in a manner similar to wild-type *L. japonicus* plants (Fig. 3.21A). The *lhk1-1 lhk1a-1*, *lhk1-1 lhk3-1*, and *lhk1a-1 lhk3-1* double mutant line were also analyzed with regard to their response to exogenously supplied BAP (Fig. 3.21B). In all cases, the double mutants carrying the *lhk1-1* mutation showed strong insensitivity to cytokinin treatment in a manner similar to the *lhk1-1* single mutant. In contrast, the *lhk1a-1 lhk3-1* double mutant displayed a wild-type response in this assay (Fig. 3.21B).

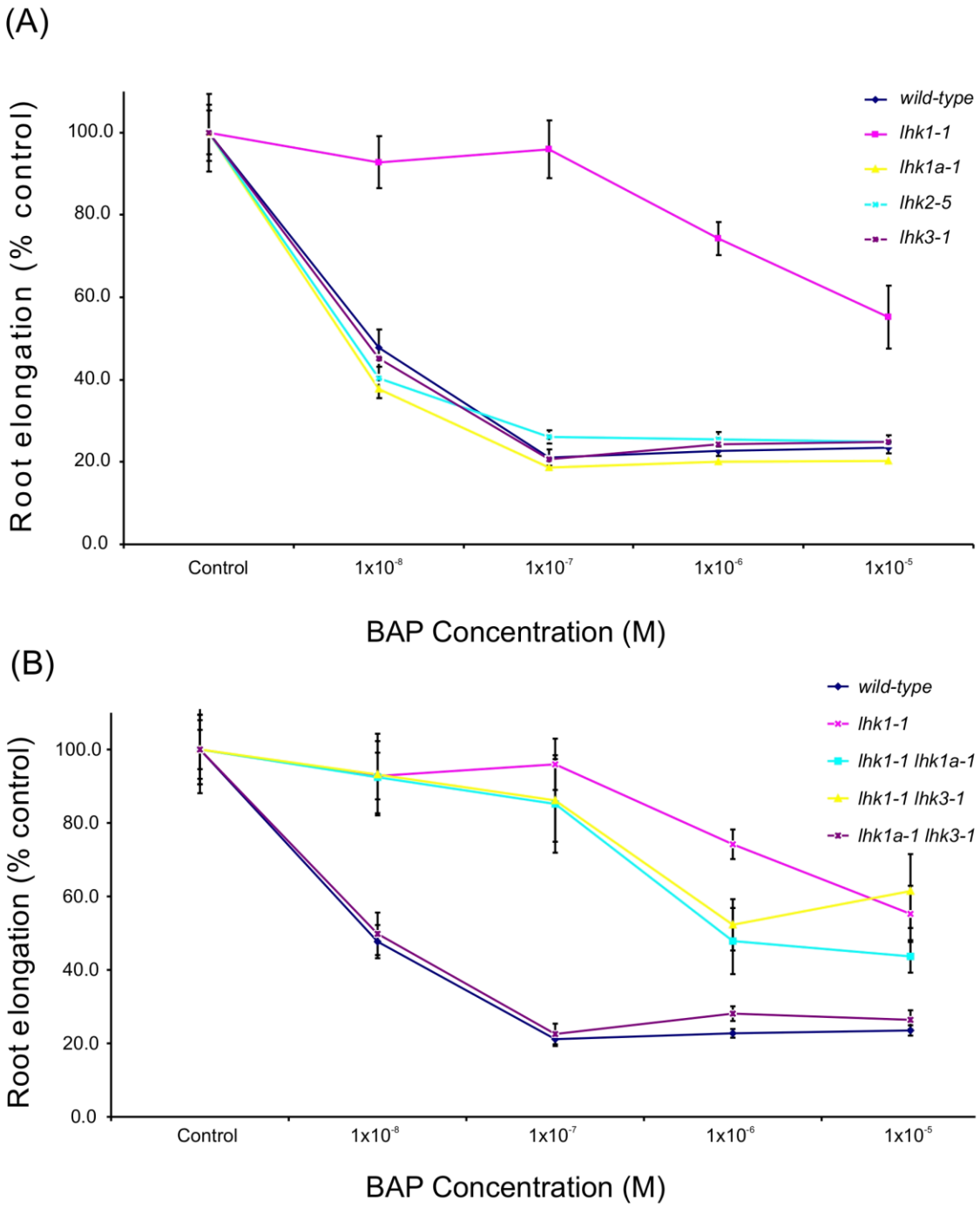


Figure 3.21 Response of the *L. japonicus* wild-type and *lhk* mutant roots to exogenous cytokinin treatment. (A) Root elongation in wild-type and *lhk* single mutants in the presence of increasing cytokinin (BAP) concentration. (B) Root elongation in wild-type and double mutant lines under the same conditions. In all cases, values represent the mean ± 95% CI ($n \geq 10$). BAP: 6-benzylaminopurine.

3.12 Characterization of *Lhk* gene promoter expression profiles in transgenic *L. japonicus* plants

The results of this study pointed to LHK1 as being uniquely responsible for mediating the cytokinin signalling during the NFS. We postulated that this could be the result of at least two mechanisms. Firstly, the seemingly unique role of cytokinin signalling through LHK1 might be a result of spatial/temporal constraints dictated by the *Lhk1* promoter. Alternatively, as yet unrecognized features of the receptor protein might dictate its involvement in signalling for root nodule primordium organogenesis. It is noteworthy that these two options are not necessarily mutually exclusive and both mechanisms might contribute to the LHK1-dependent nodule organogenesis.

To begin addressing the first option, the individual stable *L. japonicus* transgenic lines carrying each of the four *Lhk* promoters fused to the *Gus* reporter gene cassette (Fig. 3.22A) were developed via an *A. tumefaciens*-mediated transformation (see Material and Methods). Upon inoculation with *M. loti*, a portion of nodulated roots of the primary(T0) transgenic plants were collected and stained for β-glucuronidase reporter gene activity. Simultaneously, the remaining T0 plants were re-potted and allowed to self in order to produce progeny for further detailed analyses. Many of these plants although flowering profusely turned out to be partially sterile, thus hampering the progress in collecting seeds and performing subsequent study. Therefore, the results presented are based on the observations made with T0 plants only.

For *proLhk1* and *proLhk1A*, the expression profile was nearly identical in nodules. Both promoters were active in dividing cells of nodule primordia and subsequently, in the cortical cells and vascular bundles of mature nodules. Their activity was excluded from

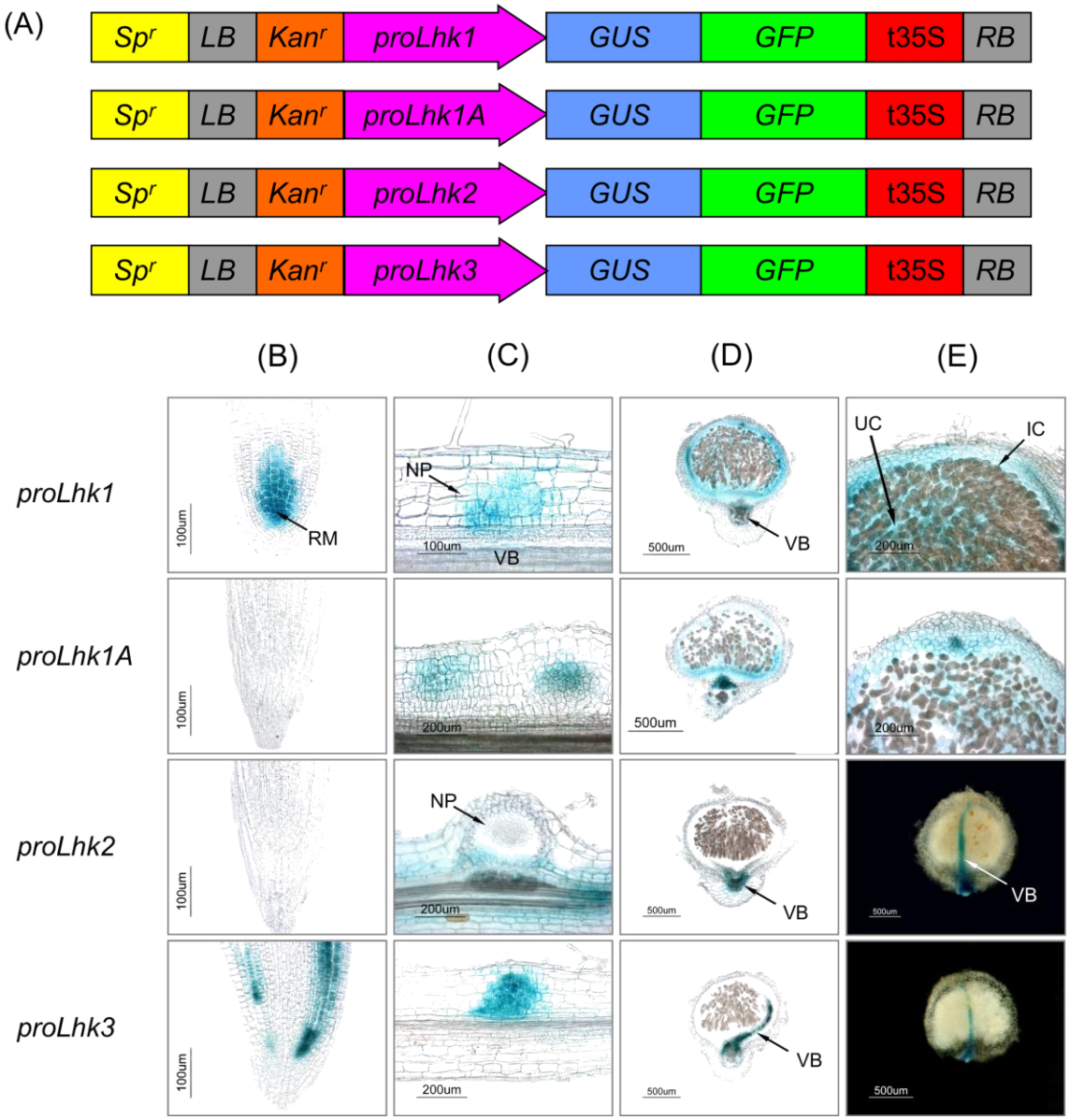


Figure 3.22 Localization of *proLhk::GUS* reporter gene activity in stable *L. japonicus* transgenic plants. (A) *proLhk* localization constructs used for stable transgenic production via *A. tumefaciens*-mediated transformation. Histochemical staining of promoter activation in sections (30µm) form the root tip (B), in young nodule primordia (C), and mature nodules (D and E). Panel (E) for *proLhk2* and *proLhk3* are whole mounts. Major tissues are highlighted including RM, root meristem; NP, nodule primordium; VB, vascular bundle; IC, infected cells; UC, uninfected cells in the top panel and throughout the remainder of the panel where the comparison is not obvious. Representative images are shown for each of the four Lhk promoters (from top to bottom; *Lhk1*, *Lhk1A*, *Lhk2*, and *Lhk3*). Sp^r, Spectinomycin resistance; LB, left border; Kan^r, kanamycin resistance; GUS, β-glucoronidase; GFP, green fluorescence protein; t35S, 35S terminator of CaMV; RB, right border.

the centrally located infected cells (i.e. these containing symbiotic bacteria) but appeared to be present in the uninfected cells. (Fig. 3.22B, D, E). However, *proLhk1::GUS* displayed a unique expression profile in the root; it was active at the root tip, being strongly associated with cells of the root meristematic region; *proLhk1A* was not expressed in this position (Fig. 3.22B).

The *proLhk2* promoter activity appeared restricted to the vasculature in all organs analyzed. Strong expression was found in the root vasculature associated with developing nodules; however, it was excluded from the dividing cells of nodule primordia and also from the root tip (Fig. 3.22B, C). In mature nodules, *proLhk2::GUS* expression was again restricted to vascular tissue of the root associated with nodules, and was also observed in vascular bundles of the nodule itself (Fig. 3.22C, D, E).

The expression profile for *proLhk3* was somewhat unique, although shared some aspects with the profile of other *Lhk* promoters. Like *proLhk1*, *proLhk3* was expressed at the root tip; however, this expression was confined to the ground tissues and was excluded from the meristematic region (Fig. 3.22B). Much like *proLhk2*, *proLhk3* expression was also restricted to the vasculature of mature nodules (Fig. 3.22D, E); however, unlike *proLhk2*, The *Lhk3* promoter-dependent expression was also observed in nodule primordia in a manner similar to *proLhk1* and *proLhk1A* (Fig. 3.22C).

3.13 Complementation of the *lhk1-1* symbiotic phenotype via overexpression of different *Lhk* cDNAs

In a second approach, complementation experiments were designed to address the question of whether or not the biochemical function of the LHK1 receptor in mediating

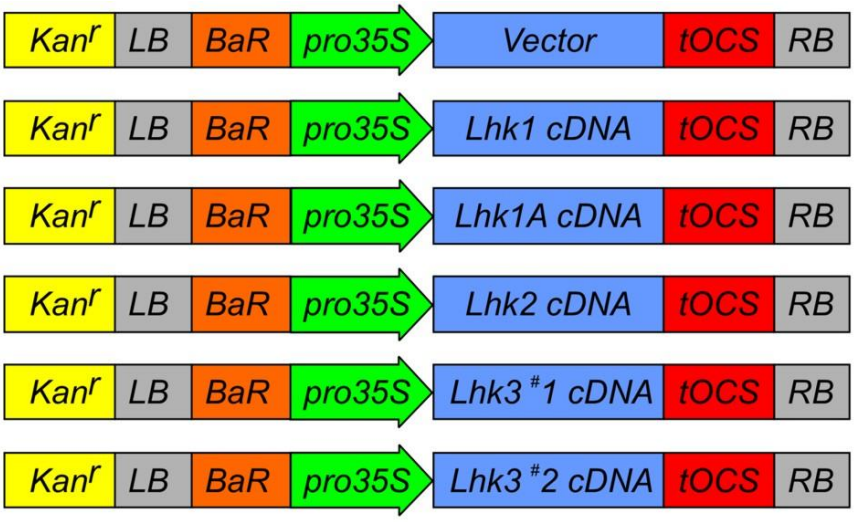
nodule organogenesis could be substituted by another cytokinin receptor. To address this question, the chimeric gene constructs carrying various *Lhk* cDNAs under the control of the constitutive *CaMV* 35S promoter were designed for *in planta* complementation experiments using *Agrobacterium rhizogenes*-mediated transformation (Petit *et al.*, 1987).

When transformed with *A. rhizogenes*, the resulting hairy roots that formed on the wild-type shoots recapitulate the wild-type nodulation phenotype providing, therefore, useful and generally accepted platform for a variety of complementation experiments that target specific root phenotypes (e.g. Groth *et al.*, 2010).

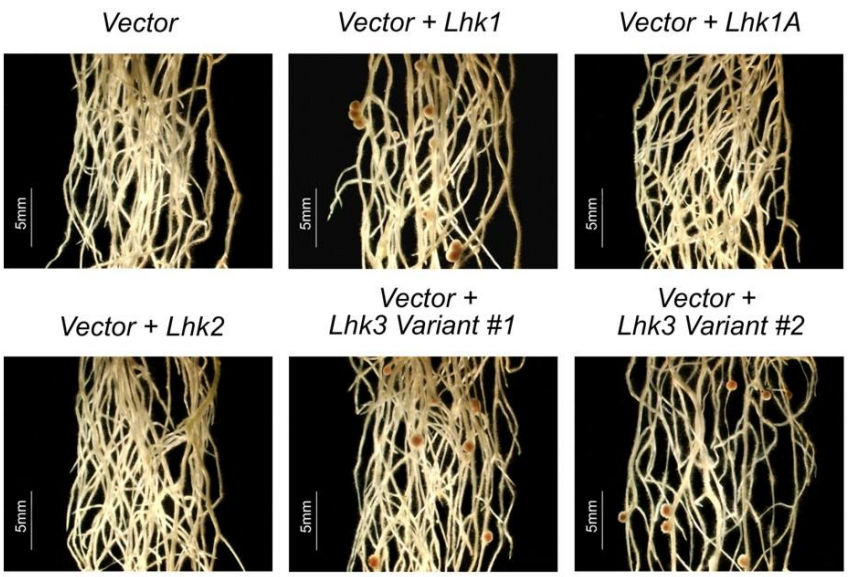
The *lhk1-1* mutant is capable of forming several aberrantly shaped nodules when inculcated with *M. loti*, although this occurs with a significant delay in comparison to wild-type plants (Murray *et al.*, 2007; see also below). However, hairy roots induced on *lhk1-1* mutant shoots exaggerate the mutant nodulation phenotype, leading to a non-nodulating (Nod^-) phenotype in this mutant genetic background (Fig. 3.23 Vector). This can be reversed by expressing the wild-type *Lhk1* cDNA, as provided to the mutant plant via an *A. rhizogenes*-mediated transformation, which results in the wild type nodulation phenotype of the resulting hairy roots (Fig. 3.23 *Vector + Lhk1*).

The same principle was used, therefore, to find out whether expression of *Lhk1A*, *Lhk2* or *Lhk3* (i.e. two *Lhk3* cDNA variants as described above), as directed by the *CaMV* 35S promoter, could restore the nodulation ability in the *lhk1-1* mutant. Interestingly, complementation of the *lhk1-1* hairy root non-nodulation phenotype was obtained for both of the *Lhk3* cDNA variants and not for *Lhk2*, or the closely-related *Lhk1A*.

(A)



(B)



(C)

	# Nodulated plants	Nodules/nodulated plant	Total # of plants
Vector	0	0	32
Vector + Lhk1	16	17.8 ± 2.0	20
Vector + Lhk1A	0	0	29
Vector + Lhk2	0	0	3
Vector + Lhk3 Variant #1	25	15.8 ± 2.7	30
Vector + Lhk3 Variant #2	10	5.8 ± 1.4	21

Figure 3.23 Complementation of the *lhk1-1* symbiotic phenotype. Various *Lhk* homologues were expressed under the control of CaMV 35S promoter in the *lhk1-1* mutant background via *A. rhizogenes*-mediated hairy-root transformation. Representative images of transgenic hairy roots are shown at 21 DAI. Quantitative scores of nodule frequency are also given. The values reported are the means ± 95% CI (n = 10) except for *Lhk2*, where only 3 plants were scored. Kan^r, kanamycin resistance; LB, left border; BaR, phosphinothricin acetyl transferase; pro35S, 35S promoter of CaMV; tOCS, terminator of octopine synthase gene; RB, right border.

3.14 Symbiotic phenotype of *lhk1-1 symRK-14* double mutants

As outlined above, the LHK1 receptor has been defined as necessary and also sufficient to mediate nodule organogenesis in *L. japonicus*. However, the *lhk1* mutants are able to form a limited number of nodules, as previously reported (Murray *et al.,* 2007 and this work). The observation that cell divisions which eventually give rise to nodule formation in *lhk1-1* mutants are initiated only after colonization of the mutant root interior by *M. loti* (the *lhk1-1* roots are hyperinfected by *M. loti* in the absence of nodule organogenesis; Murray *et al.,* 2007), might provide a plausible explanation for this apparent conundrum (for further information, see Discussion).

We hypothesised that an as yet uncharacterized signalling event originating from *M. loti* inside the root cortex, acts in an LHK1-independent manner to trigger cortical cell divisions and subsequent nodule formation in the *lhk1-1* mutant. If this is correct, blocking entry of the bacteria should completely abolish nodule formation in the *lhk1-1* mutant background. We set to test this prediction by analyzing the symbiotic phenotype of the *L. japonicus lhk1-1 symRK-14* double mutant.

In response to inoculation by *M. loti, symRK-14* develops initially empty nodules while blocking IT-dependent bacterial colonization of the roots. However, *symRK-14* nodules eventually become colonized by *M. loti* via an alternative, IT-independent mechanism, which requires the prior formation of nodule structures (S. Kosuta, M. Held and K. Szczyglowski, unpublished data). We reasoned, therefore that by incorporating the *symRK-14* mutation into the *lhk1-1* mutant background, IT-dependent hyperinfection of the *lhk1-1* root cortex (Murray *et al.,* 2007) will be prevented, thus allowing for testing the above outlined prediction.

Homozygous *lhk1-1* and *symRK-14* mutants were crossed and the resulting F1 plant was allowed to self to produce F2 progeny. The F2 segregating individuals were scored for nodulation phenotypes and also genotyped to identify their genetic status at the *Lhk1* and *SymRK* loci (i.e. the presence of wild-type or mutant allele in either homo- or heterozygote state). The double *lhk1-1 symRK-14* mutant genotype was identified in 8 out of 148 individuals tested, a frequency consistent with the predicted segregation of two unlinked loci ($\chi^2 = 0.62$, $P < 0.05$).

Unlike wild-type and single mutants of either *lhk1-1* or *symRK-14*, which all formed colonized nodules when tested three weeks after inoculation with *M. loti*, all double *lhk1-1 symRK-14* mutants showed Nod^- phenotype (Fig. 3.24D). This was in spite of the fact that bacteria were heavily accumulated at the root surface in the absence of IT formation (Fig. 3.24D); a phenotype which is reminiscent of the failed infection attempts observed in *symR-14* single mutants.

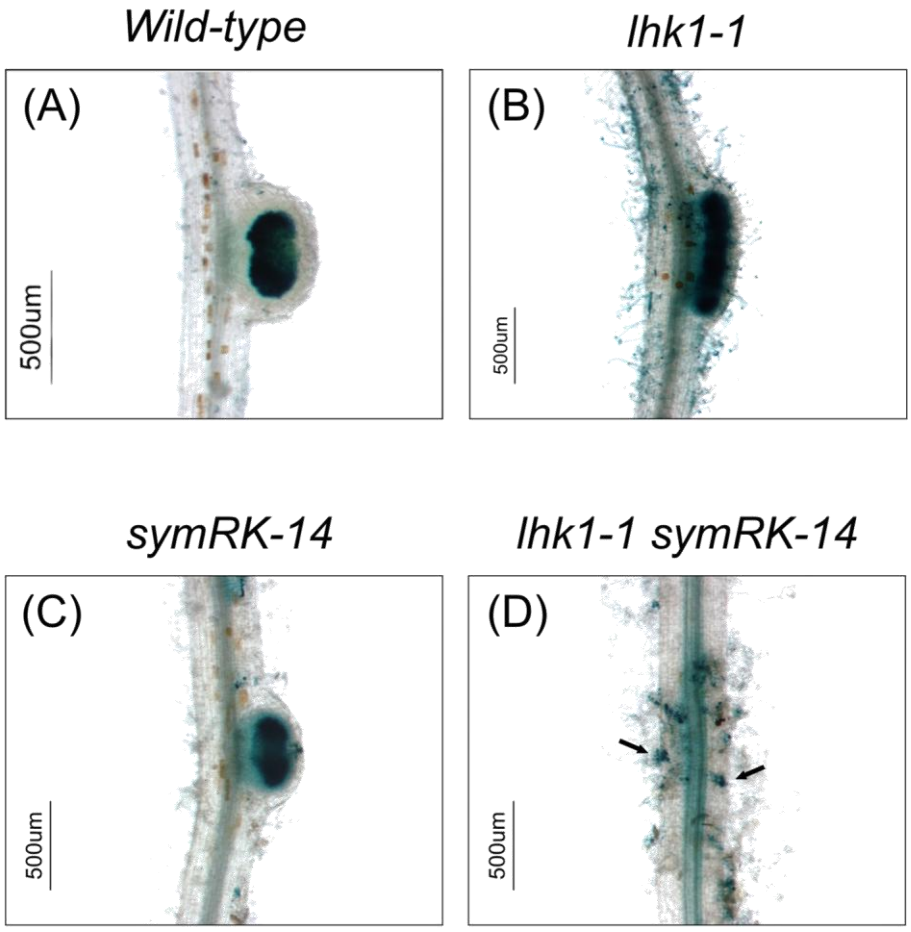


Figure 3.24 Symbiotic phenotype of the *lhk1-1 symRK-14* double mutant. Representative root sections are shown for (A) wild-type, (B) *lhk1-1*, (C) *symRK-14*, and (D) *lhk1-1 symRK-14* double mutants. Rhizobia carrying the hemA::LacZ reporter cassette are blue following staining for β-galactosidase activity. Arrowheads in (D) highlighted rhizobia attracted to the root surface in aggregates, which are unable to enter. Images are representative of the phenotype at 21 DAI with *M. loti*.

CHAPTER 4

DISCUSSION

4.1 Cytokinin and nitrogen fixing symbiosis

Cytokinins are essential plant hormones that control many aspects of plant development including cell divisions, stem-cell control in shoots and roots, growth and branching of shoots, roots and inflorescence, chloroplast biogenesis, vascular differentiation, seed development and leaf senescence (for example, Riefler *et al.*, 2006). In doing so, cytokinin signaling acts not only as a response to endogenous cues but also plays an important role as a messenger of external, environmental stimuli related to diverse biotic and abiotic stress conditions and nutrient availability (Tran *et al.*, 2007). Given this wide-range of functions, it is perhaps not surprising that cytokinin turned out to be also essential in mediating nodule organogenesis in legumes. However, the discovery that the activation of cytokinin signaling is sufficient for the formation of an entirely new organ, the root nodule, certainly provides a fascinating model for plant developmental biology. This study focused on the characterization of histidine kinase cytokinin receptors from the model legume *L. japonicus*, by analysing their role during the NFS.

4.2 The *L. japonicus* cytokinin receptor family is comprised of four members

A comprehensive search for genes encoding presumed cytokinin receptor proteins in the *L. japonicus* genome identified a small family consisting of at least four *histidine kinase receptor*-like genes, named *Lotus histidine kinase* (*Lhk*) *1*, *1A*, *2* and *3*. They were found to be localized to discrete positions in the *L. japonicus* genome. *Lhk1* and *Lhk3* were positioned on opposite ends of chromosome IV, while *Lhk1A* and *Lhk2* were placed on opposite ends of chromosome II, respectively.

Only three functional cytokinin receptors have been identified in another legume, *M. truncatula* and also in a non-legume *A. thaliana* (Gonzalez- Rizzo *et al.,* 2006; Riefler *et al.,* 2006). However, the finding that *L. japonicus* has four predicted cytokinin receptor genes was not entirely surprising. Other plants, such as rice (*Oryza sativa*), were shown to contain four members in this gene family (Du *et al.,* 2007), suggesting that the presence of four receptors in the *L. japonicus* family is not necessarily unique.

All *Lhk* genes were predicted to encode proteins that contain highly conserved domains, expected to be present in functional cytokinin receptors. In the *A. thaliana* AHK4 receptor, the CHASE domain was shown to bind cytokinin *in vitro* (Yamada *et al.,* 2001) and to be present in all characterized cytokinin receptors in *A. thaliana* (Heyl and Schmülling, 2003) and other plant species, such as maize (Yunekura-Sakakibara *et al.,* 2004). This was also the case for all LHK proteins, where the N-terminally-positioned CHASE domain was predicted to be present. The protein kinase domain and C-terminal receiver domain were also identified in all four LHK proteins and contained discrete amino-acid residues, which are highly conserved and also required for functioning of a sensor histidine kinase receptor (Werner and Schmülling, 2009). Although these data supported the notion that the identified LHK proteins indeed encode cytokinin receptors, additional functional tests were performed to verify this conclusion.

By using independent heterogonous systems in yeast and *E.coli*, cytokinin responsiveness of cells expressing the LHK1 protein was confirmed, supporting previous data (Murray *et al.,* 2007). Similarly, the imposition of cytokinin responsiveness on yeast and *E. coli* cells could be demonstrated for LHK1A and LHK3 proteins, respectively. Therefore, LHK1, LHK1A and LHK3 represent functional cytokinin receptors in *L.*

japonicus.

Unfortunately, the presence of *Lhk2* or *Lhk3* cDNAs and/or their corresponding protein products turned out to be toxic to yeast cells, precluding the use of this heterologous system. Furthermore, in spite of repeating efforts to determine LHK2 function in *E. coli*, this failed due to an apparent high instability of the expression vector containing the *Lhk2* cDNA in these cells. Thus, LHK2 remains defined as only a putative *L. japonicus* cytokinin receptor. However, while developing a working model (see below), the assumption has been made that LHK2 represents a functional cytokinin receptor in *L. japonicus* and that the *lhk2-5* mutation is deleterious to the protein function. Future investigations will have to revisit the accuracy of this assumption.

4.3 Expression patterns of the *Lhk* gene family

The expression profiles for *Lhk* genes were analyzed in major organs of *L. japonicus* and were found to be largely overlapping. Thus, mRNA corresponding to the *Lhk1*, *Lhk1A*, *Lhk2* and *Lhk3* genes were all detected in *L. japonicus* roots, nodules and leaves. Although quantification of transcripts was not performed, some tissue-preferential expression was also observed. For example, the steady-state level of *Lhk1*, *Lhk1A* and *Lhk3* transcripts appeared elevated in nodule as compared to uninoculated roots, while this was not the case for *Lhk2*. In the latter case, the majority of the transcript accumulation was detected in un-inoculated roots, with only some present in nodules and leaves.

Ubiquitous expression of the three cytokinin receptor genes across different tissues has been observed in *A. thaliana*, likely reflecting the central role these hormones

play during a wide array of developmental processes (Gonzalez-Rizzo *et al.,* 2006; Higuchi *et al.,* 2004; Nishimura *et al.,* 2004; Riefler *et al.,* 2006). The same notion is likely applicable to *L. japonicus.* However, unlike *A. thaliana*, *L. japonicus* develops root nodules, making the observations as presented in this report relevant to nodulating plant species.

Interestingly, two mRNA splicing variants were identified as being derived from the *Lhk3* locus. Alternative splicing of mRNA for kinase-type receptors is not novel (Kakita *et al.,* 2007; Lee and Yamada, 1994; Premont *et al.,* 1999; Radutoiu *et al.,* 2003; Zhukov *et al.,* 2008). However, data regarding the presence of alternative splicing in association with cytokinin histidine kinase receptor loci are scarce. The first documented observation of this type came from Inoue *et al.* (2001), who originally characterized *Ahk4* as a cytokinin receptor from *A. thaliana*. Library screening revealed two transcripts for this gene, which shared the same ORF and differed only at the 5' end of the mature mRNA due to alternative splicing in the first intron. In maize, three functional receptors have been identified and *Zmhk3* mRNA was shown to undergo alternative splicing at the 5'end, producing two functional variants (Yunekura-Sakakibara *et al.,* 2004). Although detailed characterization of the possible role(s) of these variants was not conducted, the pattern of alternative splicing observed for both *Ahk4* and *Zmhk3* is strikingly similar to *Lhk3*, thus warranting further investigation.

Corroborating the RT-PCR results, histochemical detection of the *Lhk* promoters' activity in *L. japonicus* stable transgenic lines provided a spatial resolution. Expression of the *β-glucuronidase* reporter gene activity as directed by *proLhk1*, *proLhk1A*, and *proLhk3*, was detected in the root apical meristem, root vascular bundles and a presumed

root transition, meristem to cell differentiation zone, respectively. However, reporter gene activity was absent in more mature regions of the root cortex, where nodules would be expected to form. Indeed, all three promoters were activated in the root cortex only in association with cell divisions for nodule primordia organogenesis. In contrast, the *Lhk2* promoter remained active only in root and nodule vascular bundles, but was not activated in conjunction with the cortical cell divisions.

Cumulatively, these data suggested that in addition to *Lhk1*, *Lhk1A* and *Lhk3* might partake in signaling events that initiate and/or maintain cell divisions for nodule primordia organogenesis. *Lhk2*, along with the three other *Lhk* genes might also participate in the development of nodule vascular bundles, as expression of all of these genes was detected in this tissue.

4.4 LHK1, a pivotal player during the NFS

To further assess the role of LHK receptors during symbiosis, a collection of plants carrying mutations in the *Lhk1A*, *Lhk2* and *Lhk3* loci was identified by a TILLING approach. Extensive phenotypic characterization, encompassing independent mutant lines and a variety of symbiosis-relevant developmental stages, showed no significant effects of these mutations on the symbiotic interaction. Thus, although the *lhk1a-1* and *lhk3-1* mutations were found to be deleterious to the corresponding function of these genes, infection events at the root epidermis and the number of nodules formed by mutant plants was not significantly altered in comparison to the wild-type control. Clear conclusions could not be drawn for the *lhk2-5* mutant, as the effect of this mutation on the gene function could not be determined in the heterologuous assays used (see above).

Nevertheless, similar to *lhk1A* and *lhk3* mutant lines, the *lhk2-5* mutant showed wild-type symbiotic phenotype.

These observations were consistent with the results obtained in *M. truncatula*, where RNAi-mediated silencing of the presumed orthologous functions, namely *Mthk2* (a presumed counterpart of *L. japonicus Lhk2*) and *Mthk3* (a presumed counterpart of *L. japonicus Lhk3*), did not lead to any discernible aberrations in the ability of the plant to interact symbiotically with its natural micro-symbiont, *Sinorhizobium meliloti* (Gonzalez-Rizzo *et al.*, 2006). In contrast, silencing of *Mthk1* resulted in a significant inhibition of the symbiotic process, thus recapitulating to a significant extent the mutant phenotype of the loss-of-function *lhk1-1* mutation in the *L. japonicus Lhk1* locus (Murray *et al.*, 2007; Frugier *et al.*, 2008).

These data supported, therefore, the notion that the activation of the LHK1 receptor is required and also sufficient for nodule organogenesis, which was further corroborated by the results of the double mutant analyses (i.e. analyses of combined two gene mutations among different *LHK* gene family members, see Results). At the same time, however, these observations stand in possible contradiction with the results of the histochemical analyses, which indicated a specific activation of the *Lhk1A* and *Lhk3* gene expression during nodule primordia organogenesis; therefore arguing for their involvement in the symbiotic process. This apparent conundrum is addressed further below.

4.5 The role of cytokinin receptor-dependent signaling during symbiosis

As indicated by previous work and also results presented in this thesis, the key

step in signaling for nodule primordium organogenesis in *L. japonicus* is mediated by the LHK1 cytokinin receptor (Murray *et al.,* 2007). Current data supports a notion that perception of bacterially-encoded NF by the host plant apparatus stimulates cytokinin signaling in the host plant (Gonzalez-Rizzo *et al.,* 2006). The accumulation of cytokinin in response to inoculation with nitrogen fixing bacteria has been demonstrated to occur rapidly in the root epidermis, including root hairs and subsequently, in the subtending root cortex (Oldroyd, 2007). Furthermore, cytokinin signaling genes were shown to be up-regulated by rhizobial inoculation. In *L. japonicus*, *M. loti* infection activates *Lhk1* gene expression within the root cortex (Tirichine *et al.,* 2007 and this work). Similarly, the expression of the *Mthk1* (*MtCRE1*) gene, a presumed *M. truncatula* ortholog of *Lhk1*, was shown to be upregulated by rhizobia along with several cytokinin response regulator genes that are similar to the *A. thaliana* cytokinin responsive genes, such as *ARR4* and *ARR5* (Gonzalez-Rizzo *et al.,* 2006).

In the context of a recent proposal that the activation of LHK1 is required and also sufficient for nodule organogenesis, the finding that expression of at least two additional receptor genes, namely *Lhk1A* and *Lhk3*, is also stimulated in association with nodule primordium organogenesis in *L. japonicus* was somewhat surprising. Thus, LHK1 might not be entirely unique in its function during nodule organogenesis. Could this, therefore, explain why the *L. japonicus* plants carrying a loss-of-function *lhk* allele, such as *lhk1-1*, produces a limited number of aberrant nodules? If so, why are these nodules misshaped?

Based on the results presented in this thesis, it is argued that indeed, at least partial redundancy of the LHK receptors' function might be involved in nodule

organogenesis (see below). However, the available data have not entirely ruled-out the possibility that LHK1-independent nodulation involves a mechanism that operates downstream from cytokinin signaling and thus, is cytokinin independent.

4.6 LHK1 is the sensor for exogenous cytokinin

The elongation of wild-type *L. japonicus* roots is significantly reduced in response to exogenous cytokinin treatment. In contrast, roots of *lhk1* loss-of-function mutants exhibit strong insensitivity with regard to the same treatment. Similarly, a local application of cytokinin to the surface of *L. japonicus* roots induces empty nodule structure formation in an LHK1-dependent manner, as *lhk1-1* mutants fail to form these structures (A. Heckmann and J. Stougaard, personal communication).

Importantly, *L. japonicus* plants carrying mutations in one of the three other *L. japonicus cytokinin receptor*-genes (i.e. *Lhk1A*, *2* and *3*) showed normal sensitivity to externally-applied cytokinin. Assuming that the *lhk2-5* mutation has a deleterious effect on gene function, these data would strongly support the conclusion that the external cytokinin signal is sensed primarily by LHK1. This notion is consistent with similar observations in *A. thaliana* and *M. truncatula*, where AHK4 and MtHK1 (also known as MtCRE1) presumed orthologous functions with LHK1, were defined as solely responsible for the perception of exogenous cytokinin (Gonzalez-Rizzo *et al.*, 2006; Nishimura *et al.*, 2004). Therefore, the role of LHK1 in sensing exogenously supplied cytokinin is likely to be exclusive and cannot be compensated for by any of the other LHK receptors. Although not impossible, it is important to notice that redundancy among LHK receptors in sensing exogenous cytokinin is rather unlikely, given the strong

insensitivity shown by the *lhk1* mutant roots and lack of additional effects on root elongation in the double receptor mutants that were tested.

4.7 Bacterial entry is required for LHK1-independent nodule organogenesis

In wild-type plants, the initiation of cell divisions for nodule primordia organogenesis is independent from bacterial entry into the root; this can be induced, for example, by ectopic application of NF in the absence of bacteria (van Brussel *et al.*, 1992). Furthermore, several bacterial mutants have been described, which are unable to colonize plant roots, yet remain competent in signaling for the initiation of nodule organogenesis within the root cortex (Andourel *et al.,* 1994). Therefore, a signaling event that transduces the initial perception event at the root epidermis down to the inner root cortex must be involved. Cytokinin, with its key role in this process, emerged as an excellent candidate for this function.

In contrast to wild-type plants, *lhk1* loss-of-function mutants do not initiate cell divisions upon inoculation. They are hyperinfected by *M. loti* in the absence of nodule organogenesis. The bacteria penetrate the root epidermis and descend deeper to at least the second sub-epidermal cortical layer, where they accumulate to a large extent while being confined to misguided infection threads (Murray *et al.*, 2007). Only then, in the inner cortical region which subtends extensively colonized portion of the sub-epidermal cortex, are cell divisions initiated.

We hypothesized, therefore, that bacterial entry into the subepidermal cortical region of the root might be essential for the LHK1-independent nodule organogenesis in the *lhk1-1* mutant. To test this hypothesis, the *lhk1-1 symRK-14* double mutant was

constructed. As mentioned before (see Result section), the *symRK-14* mutation aborts IT-dependent root colonization by bacteria while having no significant effect on nodule organogenesis; therefore, the hyperinfection of *lhk1-1* roots by *M. loti* was expected to be stopped in the double mutant background. Consistent with this prediction, the *lhk1-1 symRK-14* mutants showed no sign of hyperinfection in the root cortex; *M. loti* was restricted to the epidermal surface of the root. Unlike *lhk1-1* and *symRK-14* single mutants, nodule organogenesis was absent in the double mutant roots when analyzed 21 days after inoculation. This result, therefore, was consistent with the predicted need for bacterial entry into the sub-epidermal cortical region of the root for the LHK1-independent nodule organogenesis.

4.8 LHK1, not a unique receptor

The study presented in this thesis has provided evidence that both the spatial expression from the *Lhk1* promoter as well as the biochemical function of the LHK1 receptor might not be entirely unique. Firstly, expression of the *Lhk1* promoter in stable transgenic lines was found to be induced in the root cortex upon bacterial inoculation in association with nodule primordia organogenesis. However, similar expression patterns were also found for the promoters of both *Lhk1A* and *Lhk3*, but not for *Lhk2* promoter, inviting the prospect that these two genes may act at least partially redundantly with LHK1 in the inner root cortex to signal nodule primordia organogenesis.

Furthermore, *in planta* complementation of the *lhk1-1* non-nodulating phenotype in transgenic hairy roots also indicated that the biochemical function of LHK1 is not unique. One might predict *a priori* that LHK1A being the closest relative to LHK1

would be the most likely to complement the *lhk1-1* mutant phenotype; however, this turned to be the wrong prediction. The *lhk1-1* loss-of-function phenotype was complemented only by the two LHK3 protein variants. This was somewhat unexpected due to a relatively low conservation between LHK1 and the two LHK3 proteins. Nevertheless, this result indicated that the LHK1 and LHK3 proteins are biochemically interchangeable *in planta*; a finding which provides an important starting point for future comparative dissection of protein domains, as pertinent to the mediation of symbiotic signaling by these receptors (for further discussion see Perspectives). The only unique aspect of LHK1 function appeared therefore, to be its ability to sense externally applied cytokinin and also the fact that expression of the *Lhk1* promoter was localized to the root apical meristem; a pattern not recapitulated by the remaining three *Lhk* promoters.

4.9 LHK1A and LHK3 act redundantly with LHK1 in the inner root cortex

Based on the data outlined above, a working model for the LHK cytokinin receptor-dependent signaling during symbiosis is proposed (Fig. 4.1). In wild-type roots, NF perception leads to increased accumulation of cytokinin (plant-derived cytokinin, P-CK) in the root epidermis. An increase in cytokinin concentration is initially perceived by a low number of LHK1 receptors, which are constitutively present in the root epidermis and/or outer cortical cell layer and operate to sense external stimuli. This generates a feed-back regulatory mechanism, which amplifies the initial signal in a presumed cell non-autonomous manner, generating a response in subtending cortical cell layers deep inside the root (Fig. 4.1A). This is reflected in an increase in *Lhk1* and also

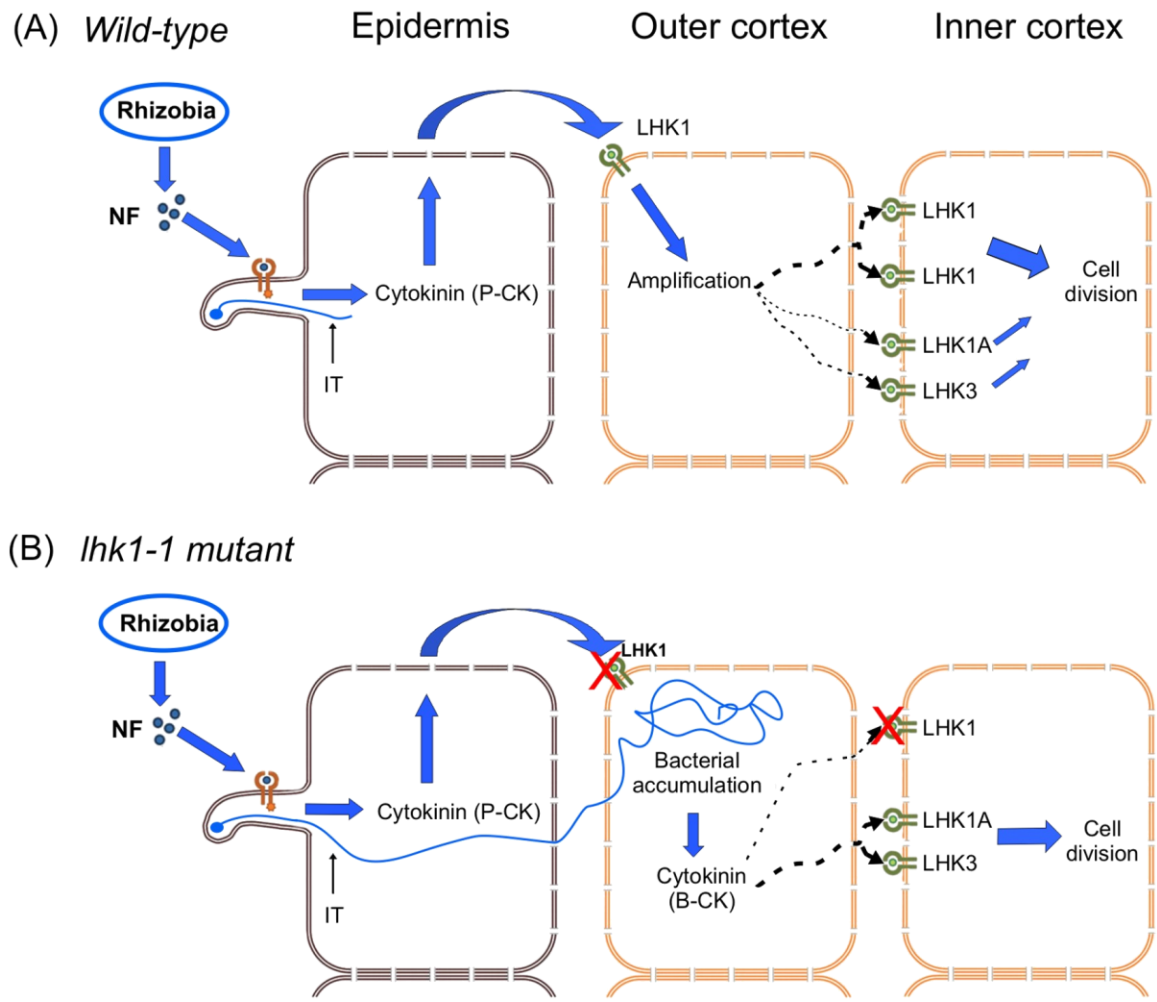


Figure 4.1 Working model for the cytokinin signaling pathway during nodule organogenesis. (A) In wild-type *L. japonicus*, rhizobially-produced NFs are perceived by NF receptors at the root epidermis. Signal transduction proceeds through common symbiosis elements leading to the intracellular production of cytokinin (P-CK), which is translocated from the epidermis to the outer cortical cells, where it is perceived exclusively by LHK1. Activation of LHK1 by cytokinin leads to the amplification of cytokinin-dependent signaling through the increased expression of the *Lhk1*, *Lhk1A* and *Lhk3* receptor genes in the inner cortical cells. This results in the increased sensitivity and initiation of cell divisions responsible for production of the nodule primordium and is primarily achieved by signalling through LHK1. (B) In *lhk1-1* mutants, P-CK is not perceived due to the lack of LHK1. This leads to the accumulation of rhizobia in the outer cortex of the root. Transmissible signals produced by these bacteria, likely in the form of cytokinin (B-CK; Oldroyd, 2007), leads to the activation of other cytokinin receptors such LHK1A and/or LHK3 acting redundantly to LHK1 in the inner root cortex.

Lhk1A and *Lhk3* expression within the inner cortical cell layers, which presumably further intensifies signaling events by increasing sensitivity of the cells to cytokinin. As the result, a threshold is reached that is necessary for stimulation of the inner cortical cells to divide, which begins nodule organogenesis.

In the absence of LHK1 (Fig. 4.1B), the increased cytokinin content in the root epidermis is not transduced to the inner cortex. Instead, bacteria enter the root without proper guidance, resulting in their accumulation along the longitudinal root axis within first and second layer of the cortical region (Murray *et al.*, 2007). Heavy accumulation of bacteria provides an alternative source of cytokinin in the root cortex, which leads to increase sensitivity, as mediated by the LHK1A and LHK3 receptors, and subsequently initiates cell divisions in the root cortex. It is tempting to speculate that bacterially-derived cytokinin (B-CK; Fig. 4.1B) and/or secretion by the bacteria of purine biosynthesis intermediates which undergo a plant-mediated conversion to biologically active cytokinin are responsible for initiation of these events. Indeed, it is known that *M. loti* synthesize cytokinin (Frugier *et al.*, 2008).

Consistent with this scenario, large regions of the root inner cortex that are positioned directly below infected cells were observed to begin divisions in *lhk1-1* mutant roots. These extended regions of cell divisions likely give rise to aberrant nodules, as described for the *lhk1-1* mutant background (Murray *et al.*, 2007). The same scenario might provide a plausible explanation how the NF-independent nodulation works in selected tropical legumes of the genus *Aeschynomene*, such as *Aeschynomene sensitiva*. What is intriguing for this group of legumes is that the nitrogen fixing *Bradyrhizobium* strain ORS278 that infect these plants do so without the canonical *nodABC* genes, which

are required for the production of NF (Giraud *et al.*, 2007). Thus, the NF-independent nodule symbiosis is exemplified by this interaction. To address the question what bacterial signals (NF, cytokinin, or others) initiate this interaction, a mutagenesis screen was conducted on *Bradyrhizobia sp.* strain ORS278, which revealed a number of mutants defective in their ability to interact with *A. sensitiva*. Surprisingly, the majority of afflicted loci in these mutants were represented by genes involved in purine biosynthesis; a major biosynthetic pathway for the production of, among others, cytokinins (Giraud *et al.*, 2007). The authors propose that cytokinin might be required for colonization of *A. sensitiva* nodules in legumes undergoing NF-independent symbiotic interactions. However, *A. sensitiva* nodules are colonized by bacteria in the IT-independent manner. They first enter the root through cracks in the root epidermis and then proceed to colonize a few sup-epidermal corticall cells, where they divide while significantly increasing their numbers. This stimulates divisions of the infected cells, which eventually give rise to fully functional, nitrogen fixing nodules (Giraud *et al.*, 2007). It is certainly intriguing to think that analogously to the postulated mechanism that mediates LHK1-idependent nodulation, the accumulation of *Bradyrhizobium* within subepidermal cortical root region leads to sufficient cytokinin production which, in turn, initiates cell division and nodule organogenesis, as seen in *A. sensitiva.*

Perspectives

Many questions regarding involvement of cytokinin signaling in the establishment of functional nitrogen fixing symbiosis remained unanswered. The work presented in this thesis, in addition to providing new insight into the organization and role of cytokinin receptors, stimulated the arrival of a new working model. Most of the predictions made by this model can now be tested, using the resource generated by this study.

A better resolution of the spatial-temporal expression of the *Lhk* genes is necessary along with the abilty to monitor *in planta* the cytokinin presence and/or redistribution within roots. Complementing this, a quantification of the *Lhk* transcripts at different stages of the symbiotic development in wild-type and also selected mutant backgrounds should be informative. Questions, such as whether or not the enhanced expression of *Lhk1A* and *Lhk3* during nodulation depends on prior activation of the LHK1 receptor should be easily addressed by these experiments. Based in the same principle, the cell-specific and subcellular localization/distribution of LHK proteins in roots should be investigated. Finally, the analysis of triple mutants, such as an *lhk1*, *lhk1A* and *lhk3* mutant, will likely be able to test the postulated redundancy in functioning of the corresponding receptors in the root inner cortex and also nodule vascular bundles, providing that such mutants will be viable and not crippled to the extent that prevents direct interpretations of the results. The ability to unambiguously define the LHK2 receptor as being responsive to cytokinin will also be important in this context.

The issue of the postulated involvement of bacterial cytokinin in nodule organogenesis remains highly controversial and will likely have to be resolved by further investigations of the NF-independent nodulation and/or used of defined mutants, such as

L. japonicus lhk1-1. The observation that LHK3 can substitute LHK1 in mediating nodule organogenesis opens up the possibility to identify feature(s) of the proteins that likely contribute to this process. Domain-swap experiments can be envisaged, which if successful, could be followed by targeted protein-protein interaction studies to identify relevant downstream interactors. This could be further extended by employing genome-wide analyses, where the entire transcriptome and proteome of the *Lhk1* gain and loss-of-function mutant lines could be analyzed through a comparative study.

Many additional experiments could be listed here. The overall challenge, however, will be to gain sufficient resolution in order to answer the key question, namely whether or not there exists a legume-specific "twist" to cytokinin signaling. A logical extension from gaining the answer to this question should be the ability to judge whether root nodules can be produced in non-legume plants and if this will help to "fix the nitrogen problem".

BIBLIOGRAPHY

Adams, D.G., and Duggan, P.S. (2008). Cyanobacteria-bryophyte symbioses. *J. Exp. Bot*. **59:** 1047-1058.

Amor, B.B., Shaw, S.L., Oldroyd, G.E.D., Maillet, F., Penmetsa, R.V., Cook, D., Long, S.R., Dénarié, J., and Gough, C. (2003). The NFP locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant J.* **34:** 495-506.

Amyot, L., and Szczyglowski, K. (2003). Symbiosis, inventiveness by recruitment? *Plant Physiol.* **131:** 935-940.

Ané, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E.D., Ayax, C., Lévy, J., Debellé, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Dénarié, J., and Cook D.R. (2004). *Medicago truncatula DMI1* Required for Bacterial and Fungal Symbioses in Legumes. *Science* **303:** 1364-1367.

Andourel, M., Demont, N., Debellé, F., Maillet, F., de Billy, F., Promé, J.-C., Dénarié, J., Truchet, G. (1994). *Rhízobíum meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. *Plant Cell* **6:** 1357-1374.

Bennett, E.M., Carpenter, S.R. and Caraco, N.R. (2001). Human impact on erodable phosphorus and eutrophication: a global perspective. *Bioscience* **51:** 227-234.

Bonfante, P., and Genre, A. (2010). Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nat. Comm.* **1:** 1-11.

Broughton, W.J., and Dilworth, M.Y. (1971). Control of leghemoglobin synthesis in snake beans. *Biochem. J.* **125:** 1075-1080.

Brundrett, M. (2004). Diversity and classification of mycorrhizal associations. *Biol. Rev.* **79:** 473-495.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., and Smith, V.N. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecol. Appl.* **8:** 559-568.

Conner, A.J., Glare, T.R., Nap, J.-P. (2003). The release of genetically modified crops into the environment. *Plant J.* **33:** 19-46.

Cooper, J.B., and Long, S.R. (1994). Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by *trans*-zeatin secretion. *Plant Cell* **6:** 215-225.

Corbesier, L., Prinsen, E., Jacqmard, A., Lejeune, P., Van Onckelen, H., Périlleux, C., and Bernier, G. (2003). Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. *J. Exp. Bot.* **54:** 2511-2517.

D'Agastino, I.B., and Kieber, J.J. (1999). Molecular mechanisms of cytokinin action. *Curr. Op. Plant Biol.* **2:** 359-364.

Das, N.K., Patau, K., and Skoog, F. (1956). Initiation of mitosis and cell division by kinetin and indoleacetic acid in excised tobacco pith tissue. *Physiol. Plant.* **9:** 640-651.

Dehio, C., and de Bruijn, F.J. (1992). he early nodulin gene *SrEnod2* from *Sesbania rostrata* is inducible by cytokinin

Diamond, J. (2002). Evolution, consequences and future of plant and animal domestication. *Nature* 418: 700-707.

Diaz, R.J., and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321:** 926-929.

Dixon, R., and Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* **2:** 621-631.

Downs, C.G., Somerfield, S.D., and Davey, M.C. (1997). Cytokinin treatment delays senescence but not sucrose loss in harvested broccoli. *Postharvest Biol. Tech.* **11:** 93-100.

Du, L., Jiao, F., Chu, J., Jin, G., Chen, M., and Wu, P. (2007). The two-component signal transduction in rice (*Oryza sativa* L.): A genome-wide study of cytokinin signal perception and transduction. *Genomics* **89:** 697-707.

Duc, G., Trouvelot, A., Gianinazzi-Pearson, V., and Gianazzi, S. (1989). First report of non-mycorrhizal plant mutants (myc-) obtained in pea *(Pisum sativum* l.) and fababean *(Vicia faba*). *Plant Sci.* **60:** 215-222.

Emery, R.J.N., Leport, L., Barton, J.E., Turner, N.C., and Atkins, C.A. (1998). *cis*-isomers of cytokinins predominate in chickpea seeds throughout their development. *Plant Physiol.* **117:** 1515-1523.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kaló, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature* **417:** 962-966.

Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D.E., and Gresshoff, P.M. (2010). Molecular analysis of legume nodule development and autoregulation. *J. Int. Plant Biol.* **52:** 61-76.

Ferreira, F.J., and Kieber, J.J. (2005). Cytokinin signalling. *Curr. Op. Plant Biol.* **8:** 518-525.

Foley, J.A., DeFries, R., Asner, G.P., Barford, C., Bonan, G., Carpenter, S.R., Chapin, F.S., Coe, M.T., Daily, G.C. Gibbs, H.K., Helkowski, J.H., Holloway, T., Howard, E.A., Kucharik, C.J., Monfreda, C., Patz, J.A., Prentice, I.C., Ramankutty, N., Snyder, P.K. (2005). Global consequences of land use. *Science* **309:** 570.574.

Flinn, J.C., De Datta, S.K., Labadan, E. (1982). An analysis of long-term rice yields in wetland soils. *Field Crops Res.* **5:** 201-216.

Frugier, F., Kosuta, S., Murray, J.D., Crespi, M., and Szczyglowski, K. (2008). Cytokinin: secret agent of symbiosis. *TIPS* **13:** 115-120.

Gage, D.J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mole. Biol. Rev. 68: 280-300.

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., and Barker, D.G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in Medicago truncatula root epidermal cells before infection. *Plant Cell* **17:** 3489-3499.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W.L., Chang, W.S., Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Verméglio, A., Médigue, C., Sadowsky, M. (2007). Legumes symbioses: absence of *Nod* genes in photosynthetic bradyrhizobia. *Science* **316:** 1307-1312.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., and Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science* **327:** 812-818.

Goley, E.D., and Welch, M.D. (2006). The ARP2/3 complex: An actin nucleator comes of age. *Nat. Rev.* **7:** 713-726.

Gonzalez-Rizzo, S., Crespi, M., and Frugier, F. (2006). The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. *Plant Cell* **18:** 2680-2693.

Graham, H.P., and Vance, C.P. (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Fields Crop Res.* **65:** 93-106.

Groth, M., Takeda, N., Perry, J., Uchida, H., Dräxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M., Wang, T.L., and Parniske, M. (2010). NENA, a *Lotus*

japonicus homolog of *Sec13*, is required for rhizodermal infection by arbuscular mycorrhizal fungi and rhizobia but dispensable for cortical endosymbiotic development. *Plant Cell (Ahead of Print)*.

Hauck R.D., and Koshino M. (1971). Slow-release and amended fertilizers. In: Olson RA *et al.* (eds) Fertilizer technology and use, pp 455-494. 2nd ed. SSSA, Madison, WI.

Heckmann, A.B., Lombardo, F., Miwa, H., Perry, J.A., Bunnewell, S., Parniske, M., Wang, T.L., and Downie, A.J. (2006). *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. *Plant Physiol.* **142:** 1739-1750.

Held, M., Hossain, Md. S., Yokota, K., Bonfante, P., Stougaard, J., and Szczyglowski, K. (2010). Common and not so common entry. *TIPS (In Press DOI:* 10.1016/j.tplants.2010.08.001 *).*

Heyl, A., and Schmulling, T. (2003). Cytokinin signal perception and transduction. *Curr. Opin. Plant Biol.* **6:** 480-488.

Higuchi, M., Pischke, M.S., Mähönen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., and Kakimoto, T. (2004). In planta functions of the Arabidopsis cytokinin receptor family. *PNAS* **101:** 8821-8826.

Hoagland, D.R., and Arnon, D.I. (1950). The water-culture method for growing plants without soil. Calif. Agic. Exp. Stn. Berekley, Circ. 347.

Huang, J., Rozelle, S.D., and Rosegrant, M.W. (1999). China's food economy in the 21st century: supply, demand, and trade. *Econ. Dev. Cult. Change* **47:** 737-766.

Imaizumi-Anraku, H., Takeda, N., Carpentier, P.M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., Pike, J., Downie, J.A., Wang, T., Sato, S., Asamizu, E., Tabata, S., Yoshikawa, M., Murooka, Y., Wu, G.J., Kawaguchi, M., Kawasaki, S., Parniske, M., Hayashi, M. (2005). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* **433:** 527-531.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* **409:** 1060-1063.

Kakita, M., Murase, K., Iwano, M., Matsumoto, T., Watanabe, M., Shiba, H., Isogai, A., and Takayama, S. (2007). Two distinct forms of M-locus protein kinase localize to the plasma membrane and interact directly with S-locus receptor kinase to transduce self-incompatibility signaling in *Brassica rapa*. *Plant Cell* **19:** 3961-3979.

Karas, B., Murray, J., Gorzelak, M., Smith, A., Sato, S., Tabata, S., and Szczyglowski, K. (2005). Invasion of *Lotus japonicus root hairless 1* by *Mesorhizobium loti* involves the nodulation factor-dependent induction of root hairs. *Plant Physiol.* **137:** 1331-1344.

Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M.H., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L., Jensen, T.H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2006). A nucleoporin is required for induction of $Ca2^{+}$ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *PNAS* **103:** 369-364.

Khush, G.S. (2001). Green revolution: the way forward. *Nat. Rev.* **2:** 815-822.

Kistner, C., and Parniske, M. (2002). Evolution of signal transduction in intracellular symbiosis. *TIPS* **11:** 511-518.

Kistner, C., Winzer, T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Webb, K.J., Szczyglowski, K., and Parniske, M. (2005). Seven symbiotic genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *Plant Cell* **17:** 2217-2229.

Lee, C.C., and Yamada, K.M. (1994). Identification of a novel type of alternative splicing of a tyrosine kinase receptor. *J. Biol. Chem.* **269:** 19457-19461.

Lev-Yadun, S., Gopher, A., and Abbo, S. (2000). The cradle of agriculture. *Science* **288:** 1602-1603.

Lohar, D.P., Schaff, J.E., Laskey, J.G., Kieber, J.J., Bilyeu, K.D., and Bird, D. McK. (2004). Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. *Plant J.* **38:** 203-214.

Madsen, L.H., Tirichine, L., Jurkiewicz, A., Sullivan, J.T., Heckmann, A.B., Bek, A. S., Ronson, C.W., James, E.K., and Stougaard, J. (2010). *Nat. Comm.* **1:** 1-12.

Maeda, T., Wurgler-Murphey, S.M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP-kinase cascade in yeast. *Nature* **369:** 242-245.

Matson, P.A., Parton, W.J., Power, A.G., and Swift, M.J. (1997). Agricultural intensification and ecosystem properties. *Science* **277:** 504-509.

Mathesius, U., Charon, C., Rolfe., B.G., Kondorosi, A., and Crespi, M. (2000). Temporal and spatial order of events during the induction of cortical cell divisions in white clover by *Rhizobium leguminosarum bv. trifolii* inoculation or localized cytokinin addition. *MPMI* **13:** 617-628.

Miller, C.O., and Skoog, F. (1953). Chemical control of bud formation in tobacco stem segments. *Amer. J. Bot.* **40:** 768-773.

Miwa, K., Ishikawa, K., Terada, K., Yamada, H., Suzuki, T., Yamashino, T., and Mizuno, T. (2007). Identification of amino acid substitutions that render the *Arabidopsis* cytokinin receptor histidine kinase *AHK4* constitutively active. *Plant Cell Phyisol.* **48:** 1809-1814.

Miyahara, A., Richens, J., Starker, C., Morieri, G., Smith, L., Long, S., Downie, J.A., and Oldroyd, G.E.D. (2010). Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in *Medicago truncatula*. *MPMI* (*In Press DOI*: 10.1094/MPMI-06-10-0144).

Mok, D.W.S., and Mok, M.C. (Eds.). "Cytokinins: chemistry, activity, and function." CRC press, Florida, USA, 1994. pp 113-125.

Müller, B., and Sheen, J. (2007). Advances in cytokinin signalling. *Science* **318:** 68-69.

Murray, J., Karas, B., Ross, L., Brachmann, A., Wagg, C., Geil, R., Perry, J., Nowakowski, K., MacGillivary, M., Held, M., Stuogaard, J., Peterson, L., Parniske, M., and Szczyglowski, K. (2006) Genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype. *MPMI* **19**: 1082-1091.

Murray, J.D., Karas, B.J., Sato, S., Tabata, S., Amyot, L., and Szczyglowski, K. (2007). A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. *Science* **315:** 101-104.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. *Plant Cell* **16:** 1365-1377.

Niwa, S., Kawaguchi, M., Imaizumi-Anraku, H., Chechetka, S.A., Ishizaka, M., Ikuta, A., Kouchi, H. (2001). Responses of a model legume *Lotus japonicus* to lipochitin oligosaccharide nodulation factors purified from *Mesorhizobium loti* JRL501. *MPMI* **14:** 848-856.

Oldroyd, G.E.D., and Long, S.R. (2003). Identification and Characterization of *Nodulation-Signaling Pathway 2*, a Gene of *Medicago truncatula* Involved in Nod Factor Signaling. *Plant Phsyiol.* **131:** 1027-1032.

Oldroyd, G.E.D. (2007). Nodules and hormones. *Science* **315:** 52-53.

Oldroyd, G.E.D., and Downie, A.J. (2008). Coordinating nodule development morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* **59:** 519-546.

Papendick, R.I., Elliot, L.F., and Dahlgren, R.B. (1986). Environmental consequences of modern production agriculture: how can alternative agriculture address these issues and concerns? *Amer. J. Alt. Agri.* **1:** 3-10.

Parkinson, J.S., and Kofoid, E.C. (1992). Communication modules in bacterial signalling proteins. *Annu. Rev. Genet.* **26:** 71-112.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Rev. Microbiol.* **6:** 763-775.

Pawlowski, K., and Sirrenberg, A. (2003). Symbiosis between *Frankia* and actinorhizal plants: Root nodules of non-legumes. *Ind. J. Exp. Bot.* **41:** 1-19.

Perilli, S., Moubayidin, L., Sabatini, S. (2010). The molecular basis of cytokinin function. *Curr. Opin. Plant Biol.* **13:** 21-26.

Perry, J.A., Wang, T.L., Welham, T.J., Gardner, S., Pike, J.M., Yoshida, S., and Parniske, M. (2003). A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. *Plant Physiol.* **131:** 866-871.

Petit, A., Stougaard, J., Kuhle, A., Marcker, K.A., and Tempe, J. (1987). Transformation and regeneration of the legume *Lotus corniculatus*: A system for molecular studies of symbiotic nitrogen fixation. *Mol. Gen. Genet.* **207:** 245-250.

Premont, R.T., Macrae, A.D., Aparicio, A.J.R., Kendall, H.E., Welch, J.E., and Lefkowitz, R.J. (1999). The GRK4 family of G protein-coupled receptor kinases. *J. Biol. Chem.* **274:** 21381-29389.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Grønlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425:** 585-591.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40-54.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczyglowski, K., Downie, J.A., Parniske, M., Hayashi, M., and Kawaguchi, M. (2007). *NUCLEOPORIN85* required for calcium spiking, fungal and bacterial symbiosis, and seed production in *Lotus japonicus*. *Plant Cell* **19:** 610-624.

Sato, S., Nakamura, N., Kaneko, N., Asamizu, E., Kato, T., Nakao, M., Sasamoto, S., Watanabe, A., Ono, A., Kawashima, K., Fujishiro, T., Katoh, M., Kohara, M.,

Kishida, Y., Minami, C., Nakayama, S., Nakazaki, N., Shimizu, Y., Shinpo, S., Takahashi, C., Wada, T., Yamada, M., Ohmido, N., Hayashi, M., Fukui, K., Baba, T., Nakamichi, T., Mori, H., and Tabata, S. (2008). Genome structure of the legume, *Lotus japonicus*. *DNA Res.* **15:** 227-239.

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic nodules. *Nature* **402:** 191-195.

Schindler, D.W. (1977). Evolution of phosphorus limitation in lakes. *Science* **195:** 260-262.

Schmülling, T. (2001). CREam of cytokinin signalling: receptor identified. *TIPS* **6:** 281-284.

Smale, M. (1997). The green revolution and wheat genetic diversity: some unfounded assumptions. *World Devel.* **25:** 1257-1269.

Smith, B.E. (2002). Nitrogenase reveals its inner secrets. *Science* **297:** 1654-1655.

Sprent, J.I., and James, E.K. (2007). Legume evolution: where do nodules and mycorrhizas fit in? *Plant Physiol.* **144:** 575-581.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. *Annu. Rev. Biochem.* **69:** 183-215.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczyglowski, K., and Parniske, M. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* **417:** 959-966.

Sturtevant, D.B., and Taller, B.J. (1989). Cytokinin production by *Bradyrhizobium japonicum*. *Plant Physiol.* **89:** 1247-1252.

Szczyglowski, K., and Stougaard, J. (2008). Lotus genome: pot of gold for legume research. *TIPS* **13:** 515-517.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* **418:** 671-677.

Tirichine, L., James, E.K., Sandal, N., and Stougaard, J. (2006). Spontaneous root-nodule formation in the model legume *Lotus japonicus*: a novel class of mutants nodulates in the absence of rhizobia. *MPMI* **19:** 373-382.

Tirichine, L., Sandal, N., Madsen, L.H., Radutoiu, S., Albrektsen, A.S., Sato, S., Asamizu, E., Tabata, S., and Stougaard, J. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* **315:** 104-107.

Toh, J.P.C., Deruère, J., Maxwell, B.D., Morris, V.F., Hutchinson, C.E., Ferreira, F.J., Schaller, G.E., Kieber, J.J. (2007). *Plant Cell* **19:** 3901-3914.

Tran, L.S.P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., Yamaguchi-Shinozaki, K. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*.

Ueguchi, C., Koizumi, H., Suzuki, T., and Mizuno, T. (2001). Novel family of sensor histidine kinase genes in *Arabidopsis thaliana*. *Plant Cell Physiol.* **42:** 231-235.

van Brussel, A.A., Bakhuizen, R., van Spronzen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J., and Kijne, J.W. (1992). Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. Science 257: 70-72.

Vance, C.P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiol.* **127:** 390-397.

Vance, C.P., Uhde-Stone, C., and Allan, D.L. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol.* **157:** 423-447.

Vierheilig, H., Coughlan, A.P., Wyss, U., and Piché, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl. Environ. Microbiol.* **64:** 5004-5007.

Vitousek, P. M. and P. A. Matson (1993). Agriculture, the global nitrogen cycle, and trace gas flux. The Biogeochemistry of Global Change: Radiative Trace Gases. R. S. Oremland. New York, Chapman and Hall: 193-208.

Werner, T., and Schmulling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* **12:** 527-538.

Werner, T., Motyka, V., Strnad, M., and Schmulling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristems activity. *Plant Cell* **15:** 2532-2550.

Wopereis, J., Pajuelo, E., Dazzo, F.B., Jiang, Q.Y., Gresshoff, P.M., de Bruijn, F.J., Stougaard, J., and Szczyglowski, K. (2000). Short root mutant of Lotus japonicus with a dramatically altered symbiotic phenotype. *Plant J.* **23:** 97-114.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., Mizuno, T. (2001). The *Arbidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* **42:** 1017-1023.

Yano, K., Yoshida, S., Muller, J., Singh, S., Banba, M., Vickers, K., Markmann, K., White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T.L., Kawaguchi, M., Inaizumi-Anraku, H., Hayashi, M., and Parniske, M. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. *PNAS* **105:** 20540-20545.

Yokota, K., Fukai, E., Madsen, L.H., Jurkiewicz, A., Rueda, P., Radutoiu, S., Held, M., Hossain, M.D.S., Szczyglowski, K., Morieri, G., Oldroyd, G.E.D., Downie,J.A., Nielsen, M.W., Rusek, A.M., Sato, S., Tabata, S., James, E.K., Oyaizu, H., Sandal, N., and Stougaard, J. (2009). Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. *The Plant Cell* **21:** 267-284.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., and Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and responses to cis-zeatin. *Plant Physiol.* **134:** 1654-1661.

Young, N.D., and Udvardi, M. (2009). Translating *Medicago* genomics to crop legumes. *Curr. Opin. Plant Biol.* **12:** 193-201.

Zhukov, V., Radutoiu, S., Madsen, L.H., Rychagova, T., Ovchinnikova, E., Borizov, A., Tikhonovich, I., and Stougaard, J. (2008). The pea sym37 receptor kinase gene controls infection-thread initiation and nodule development. *MPMI* **21:** 1600-1608.

APPENDIX I

Research contributions not included in the thesis

In addition to data presented in this thesis, the following contributions were made by M. Held:

1. Development of the *har1-1 MG20* introgression for the map-based cloning of *L. japonicus* mutants displaying subtle symbiotic phenotypes (Murray *et al.*, 2006; *Molecular Plant-Microbe Interactions* **19**: 1082-1091).

2. Extensive phenotypic characterization of the *nap* and *pir* mutants of *L. japonicus* (Yokoda *et al.*, 2009; *Plant Cell* **21**: 267-284).

3. Intellectual review of the genetic mechanisms regulating bacterial entry, the NFS and AM symbiosis of legume plants (Held *et al.*, 2010; *TIPS DOI* 10.1016/j.tplants.2010.08.001).

4. Detailed analysis of the *symRK-14* mutant (Kosuta *et al., in preparation;* '*Lotus japonicus symRK-14* uncouples epidermal calcium spiking from nodule primordia organogenesis').

5. Mapping and phenotypic analysis of several symbiotic mutants of *L. japonicus* using the *har1-1 MG20* introgression line (Hossain, M.D.S., Held, M., and Szczyglowski, K. *unpublished data*).

CURRICULUM VITAE

Mark Anton Held

Essential Qualifications

Education

University of Western Ontario
London, ON, Canada
2006-present, PhD. Candidate
Academic Record to date: 92.5% overall

University of Waterloo
Waterloo, ON, Canada
2003-2005, MSc.

Trent University
Peterborough, ON, Canada
1999-2003, HBSc.

Scholarships/Distinctions

2008-2010: Natural Science and Engineering Research Council of Canada, PGS-D2 ($42,000). This highly competitive, federal-level grant is awarded to PhD students based on research potential, academic excellence, and publication record.

2010: J.D. Detweiler award in plant sciences ($1000). This institutional level honour is awarded to a single PhD student for demonstrated, research-based excellence in a relevant plant sciences field.

2009: Graduate student travel award ($500). This institutional travel award was given to me by the Biology Department at the University of Western Ontario on research merit. The award was used to supplement my travel to the 2009 Model Legume Congress in Asilomar, California, USA.

2009: 2009 Model Legume Congress student travel award ($500). This international travel award was awarded to me on research merit and was used to supplement my travel to the 2009 Model Legume Congress in Asilomar, California, USA.

2008: Ontario Graduate Scholarship ($15,000) – declined. This highly competitive, provincial grant is awarded to MSc or PhD students based on research potential, academic excellence, and publication record. The award was declined because I had received an NSERC PGS-D2 award at the same time.

2007-2008: Ontario Graduate Scholarship ($15,000). This highly competitive, provincial grant is awarded to MSc or PhD students based on research potential, academic excellence, and publication record.

2006-Present: Western Graduate Research Scholarship ($13,400). This institutional award is given to graduate students by the University of Western Ontario based on academic excellence.

2005: E.B. Dumbroff Award awarded for the top graduate-level thesis in plant sciences ($250). This institutional award is given to the student with the top, graduate-level thesis in plant sciences. It is awarded by the Biology Department at the University of Waterloo.

2003-2005: University of Waterloo Graduate Research Scholarship ($5,500). This institutional award is given to graduate students by the University of Waterloo based on academic excellence.

Publications

a) Articles published or accepted

Held, M., Hossain, MD. S., Yokota, K., Bonfante, P., Stougaard, J., and Szczyglowski, K. **(2010)**. Common and not so common symbiotic entry. (*In Press: Trends in Plant Science; DOI* 10.1016/j.tplants.2010.08.001) (Ph.D. work).

Yokota, K., Fukai, E., Madsen, L.H., Jurkiewicz, A., Rueda, P., Radutoiu, S., **Held, M.,** et al. **(2009)** Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. *The Plant Cell* **21**: 267-284 (Ph.D. work).

Held, M., Pepper, A., Smith, M.D., Emery, R.J.N. and Guinel, F.C. **(2008)** The pea (*Pisum sativum* L.) nodulation mutant R50 (*sym16*) displays altered activity and expression profiles for cytokinin oxidase/dehydrogenase. *Journal of Plant Growth Regulation* **27**: 170-180 (MSc. work).

Murray, J., Karas, B., Ross, L., Brachmann, A., Wagg, C., Geil, R., Perry, J., Nowakowski, K., MacGillivary, M., **Held, M.**, et al. **(2006)** Genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype. *Molecular Plant Microbe Interactions* **19**: 1082-1091 (Ph.D. work).

Held, M.A., Quesnelle, P.E., and Emery, R.J.N. **(2005)** Seasonal Changes of Cytokinins in Upper and Lower Leaves of a Sugar Maple Crown. *Biologia Plantarum* **49**: 455-458 (HBSc. Work).

b) Articles submitted

Kosuta, S., **Held, M.,** MacGillivary, M., Morieri, G., Oldroyd, G., Downie, A., Johansen, C., Hossain, MD. S., Karas, B., and Szczyglowski, K. **(2009)**. *Lotus japonicus symRK-14* uncouples epidermal calcium spiking from nodule primordia organogenesis (*Submitted to Plant Journal*, *Manuscript* #TPJ-01038-2009*; to be re-submitted with revisions*) (Ph.D. work).

Experience

University of Western Ontario:

2006-present: Research Assistant (full-time).
2006-present: Teaching Assistant (full-time).

University of Waterloo:

2003-2005: Research Assistant (full-time).
2003-2005: Teaching Assistant (full-time).

Presentations - Oral

Held, M. (2009). Genetic Mechanisms Regulating the Nitrogen Fixing Symbiosis in *Lotus japonicus*. University of Western Ontario, London, Ontario, Canada (Ph.D. work).

Held, M. (2007). Investigating the Molecular Genetic Mechanisms Regulating Root Colonization in *Lotus japonicus* by *Mesorhizobium loti*. University of Western Ontario, London, Ontario, Canada (Ph.D. work).

Held, M.A., and Guinel, F.C. **(2005).** Characterizing cytokinin oxidase throughout the development of R50 (*sym 16*), a pea mutant accumulating cytokinins. Plant Canada 2005, Edmonton, Alberta, Canada (MSc. work).

Held, M.A., and Guinel, F.C. **(2004).** The biochemical properties of cytokinin oxidase in the pleiotropic mutant R50 (*sym 16*). Canadian Society of Plant Physiologists Annual Meeting, Univ. of Guelph, Ontario, Canada (MSc. work).

Co-authored presentations (the presenter is denoted by an asterisk)

*Szczyglowski, K., **Held, M.,** Hossain, M., Karas, B., Kosuta, S., and Ross, L. **(2009)**. Common and not so common keys to symbiotic entry. XIV Congress of the International Society for Plant-Microbe interactions, Quebec City, Quebec, Canada (PhD work).

*Szczyglowski, K., Kosuta, S., Karas, B., and **Held, M.** (**2007)**. "Cytokinins in symbiosis: new insight into an old hypothesis". Canadian Society of Plant Physiologists Eastern Regional Meeting, University of Western Ontario, London, Ontario, Canada (PhD work).

*Szczyglowski, K., Kosuta, S., **Held, M.,** Karas, B. **(2007)**. "Cytokinin, Secret agent of Nodulation". The 17th Annual Meeting of Japanese Society of Plant Microbe Interactions and International Symposium of Frontier Science Research Center, Kagoshima University, Japan (PhD work).

Kosuta, S., MacGillivary, A., **Held, M.**, Johansen, C., Karas, B., and *Szczyglowski, K. **(2007)**. "*symRK-14*, a new allele with attitude". The 20th North American Symbiotic Nitrogen Fixation Conference, Milwaukee, Wisconsin, USA (PhD work).

*Szczyglowski, K**.,** Murray, J., Karas, B., **Held, M.**, Amyot, L., and Kosuta S. **(2007)**. "Lipochitin-oligosaccharides and cytokinin give plants the nod". The 19th Annual Meeting of the International Plant Growth Substances Association, Puerto Vallarta, Mexico (PhD work).

Karas, B., Murray, J., **Held, M.**, Ross, L., Amyot, L., and *Szczyglowski, K. **(2006)**. "Symbiotic roots of legume genomes". 4th Canadian Plant Genomics Workshop, Ottawa, Canada (PhD work).

Poster presentations

Held, M., Huynh, C., Perry, J., Wang, T., and Szczyglowski, K. **(2010)**. Cytokinin signaling and nodule organogenesis in *Lotus japonicus*. The 21st North American Symbiotic Nitrogen Fixation Conference, Columbia, Missouri, USA (PhD work).

Hossain, MD S., **Held, M.,** Ross, L., Sato, S., Tabata, S., Karas, B., and Szczyglowski, K. **(2009)**. Novel plant loci required for rhizobium accommodation and symbiosis. XIV Congress of the International Society for Plant-Microbe interactions, Quebec City, Quebec, Canada (PhD work).

Held, M., Johansen, C., Perry, J., Wang, T. and Szczyglowski, K. **(2009)**. Characterization of the *Lotus japonicus* cytokinin receptor gene family and their role in symbiosis. 2009 Model Legume Congress, Asilomar, California, USA (PhD work).

Held, M., Kosuta, S., Amyot, L., and Szczyglowski, K. **(2007)**. Role of cytokinin and *NIN* transcriptional regulator in colonization of roots by nitrogen fixing bacteria. CSPP Eastern Regional Meeting, London, Ontario, Canada (PhD work).

Szczyglowski, K., Kosuta, S., **Held, M.** *et al.* **(2007).** Cytokinin, Secret agent of Nodulation. The Molecular Aspect of Plant-Microbe Interactions and Plant Immunity as a Clue to Enhance Plant Ability, Kagoshima, Japan (PhD work).

Guinel, F.C., Ferguson, B.J., **Held, M.A.** *et al.* **(2007).** R50 (*sym16*): a pea nodulation mutant with a shoot not responding to cytokinins? The XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento, Italy (MSc work).

Kosuta, S., Karas, B., **Held, M.** *et al.* **(2007).** symRK-14: intracellular symbiosis reveals its evolutionary roots. The XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento, Italy (PhD work).

Sloetjes, L., Pepper, A., Morse, A., **Held, M.A.** *et al.* **(2006).** The pleiotropic pea mutant R50, a low nodulator with a complex hormonal phenotype. Conférence Sève, Montréal, Canada (MSc work).

Held, M.A., and Guinel, F.C. **(2005).** Cytokinin oxidase activity throughout the development of R50, a pea mutant accumulating cytokinins. XVII International Botanical Congress, Vienna, Austria (MSc work).

Held, M.A., Quesnelle, P., and Emery, R.J.N. **(2002)** Seasonal Changes of Cytokinins in Upper and Lower Leaves of a Sugar Maple Crown. CSPP/SCPV Easter Regional Meeting, Brock Univ., St. Catherines, Ontario, Canada (HBSc work).